

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

From the INTERNATIONAL BUREAU

To:

Assistant Commissioner for Patents  
United States Patent and Trademark  
Office  
Box PCT  
Washington, D.C. 20231  
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing:

03 August 2000 (03.08.00)

International application No.:

PCT/GB00/00263

Applicant's or agent's file reference:

SCB/52068001

International filing date:

28 January 2000 (28.01.00)

Priority date:

28 January 1999 (28.01.99)

Applicant:

BRAMLEY, Peter, Michael et al

1. The designated Office is hereby notified of its election made:



in the demand filed with the International preliminary Examining Authority on:

02 June 2000 (02.06.00)



in a notice effecting later election filed with the International Bureau on:

2. The election ☒ was



was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO  
34, chemin des Colombettes  
1211 Geneva 20, Switzerland

Facsimile No.: (41-22) 740.14.35

Authorized officer:

J. Zahra

Telephone No.: (41-22) 338.83.38

## TENT COOPERATION TREATY

## PCT

## INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference <b>SCB/52068001</b>	<b>FOR FURTHER ACTION</b> see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. <b>PCT/GB 00/ 00263</b>	International filing date (day/month/year) <b>28/01/2000</b>	(Earliest) Priority Date (day/month/year) <b>28/01/1999</b>
Applicant <b>ROYAL HOLLOWAY AND BEDFORD NEW COLLEGE et al.</b>		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 4 sheets.

☒ It is also accompanied by a copy of each prior art document cited in this report.

## 1. Basis of the report

- a. With regard to the **language**, the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

☐ the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

- b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing:

☐ contained in the international application in written form.

☐ filed together with the international application in computer readable form.

☒ furnished subsequently to this Authority in written form.

☒ furnished subsequently to this Authority in computer readable form.

☒ the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

☒ the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. ☐ **Certain claims were found unsearchable** (See Box I).

3. ☐ **Unity of Invention is lacking** (see Box II).

4. With regard to the **title**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established by this Authority to read as follows:

5. With regard to the **abstract**,

☒ the text is approved as submitted by the applicant.

☐ the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No. \_\_\_\_\_

☐ as suggested by the applicant.

☐ because the applicant failed to suggest a figure.

☐ because this figure better characterizes the invention.

☐ None of the figures.

## INTERNATIONAL SEARCH REPORT

national Application No

/GB 00/00263

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/54 C12N9/10 C12N1/21 A01H5/00 C12N15/82  
C12Q1/48 //(C12N1/21,C12R1:19)

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A01H C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>LOIS LUISA MARIA ET AL: "Cloning and characterization of a gene from Escherichia coli encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxyxylulose 5-phosphate, a common precursor for isoprenoid, thiamin, and pyridoxol biosynthesis."</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA MARCH 3, 1998, vol. 95, no. 5, 3 March 1998 (1998-03-03), pages 2105-2110, XP002116673 ISSN: 0027-8424 cited in the application the whole document</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	1-5,7,8, 11-13,25



Further documents are listed in the continuation of box C.



Patent family members are listed in annex.

## \* Special categories of cited documents :

- "A" document defining the general state of the art which is not considered to be of particular relevance
- "E" earlier document but published on or after the international filing date
- "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

9 June 2000

Date of mailing of the international search report

28/06/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Authorized officer

Holtorf, S

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>LANGE ET AL: "A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway"</p> <p>FASEB JOURNAL, US, FED. OF AMERICAN SOC. FOR EXPERIMENTAL BIOLOGY, BETHESDA, MD, vol. 95, March 1998 (1998-03), pages 2100-2104, XP002116672</p> <p>ISSN: 0892-6638</p> <p>cited in the application</p> <p>the whole document</p> <p>---</p>	1-35
A	<p>LICHTENTHALER HARTMUT K ET AL:</p> <p>"Biosynthesis of isoprenoids in higher plant chloroplasts proceeds via a mevalonate-independent pathways."</p> <p>FEBS LETTERS 1997, vol. 400, no. 3, 1997, pages 271-274, XP002139893</p> <p>ISSN: 0014-5793</p> <p>cited in the application</p> <p>the whole document</p> <p>---</p>	1-35
A	<p>MANDEL A ET AL: "CLA1, a novel gene required for chloroplast development, is highly conserved in evolution"</p> <p>THE PLANT JOURNAL, vol. 9, no. 5, May 1996 (1996-05), pages 649-658-658, XP002122907</p> <p>cited in the application</p> <p>the whole document</p> <p>---</p>	1-35
A	<p>KANEKO, T., ET AL. : "sequence analysis of the genome of the unicellular cynaobacterium Synechocystis sp. strain PCC6803. II. sequence determination of the entire genome and assignment of potential protein-coding regions"</p> <p>EMBL SEQUENCE DATA LIBRARY, 15 July 1998 (1998-07-15), XP002139910</p> <p>heidelberg, germany</p> <p>accession no. P73067</p> <p>---</p>	1-35
A	<p>KOBAYASHI, Y., ET AL. : "untitled"</p> <p>EMBL SEQUENCE DATA LIBRARY, 1 October 1996 (1996-10-01), XP002139911</p> <p>heidelberg, germany</p> <p>accession no. P54523</p> <p>---</p>	1-35
P,X	<p>WO 99 58649 A (UNIV MARYLAND ;DELLAPENNA DEAN (US); MOEHS CHARLES P (US); CUNNING)</p> <p>18 November 1999 (1999-11-18)</p> <p>the whole document</p> <p>---</p>	1-16,25, 27-30
	<p>---</p> <p>-/--</p>	

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P, X	WO 99 11757 A (MCCASKILL DAVID G ; LANGE BERND M (US); UNIV WASHINGTON (US); WILDU) 11 March 1999 (1999-03-11)  the whole document ---	1-5, 7-13, 17-25, 28, 30
P, X	HARKER M ET AL: "Expression of prokaryotic 1-deoxy-D-xylulose-5-phosphatases in Escherichia coli increases carotenoid and ubiquinone biosynthesis." FEBS LETTERS APRIL 1, 1999, vol. 448, no. 1, 1 April 1999 (1999-04-01), pages 115-119, XP002139894/ ISSN: 0014-5793 the whole document ---	1-3, 5, 7-13, 25-30
P, X	WO 99 52938 A (HASSAN JOMAA) 21 October 1999 (1999-10-21) the whole document ---	17-24
P, X	DE 298 00 547 U (HOECHST SCHERING AGREVO GMBH) 20 May 1999 (1999-05-20)  the whole document ---	1-5, 7-13, 16, 18-25, 28, 30, 31
P, X	WO 99 53071 A (KUZUYAMA TOMOHISA ; MIYAKE KOICHIRO (JP); OZAKI AKIO (JP); SETO HAR) 21 October 1999 (1999-10-21)  the whole document ---	1-5, 7-13, 17, 19-25, 27-31, 33-35
E	WO 00 08169 A (EBNETH MARCUS ; HERBERS KARIN (DE); REINDL ANDREAS (DE); SUNGENE GM) 17 February 2000 (2000-02-17) the whole document -----	1-25, 27-31, 33-35

# INTERNATIONAL SEARCH REPORT

I tion on patent family members

International Application No

T/GB 00/00263

Patent document cited in search report		Publication date	Patent family member(s)		Publication date
WO 9958649	A	18-11-1999	AU 3964599	A	29-11-1999
WO 9911757	A	11-03-1999	AU 8925898	A	22-03-1999
WO 9952938	A	21-10-1999	DE 19825585	A	21-10-1999
			DE 19828097	A	30-12-1999
			DE 19831637	A	27-01-2000
			DE 19831639	C	11-05-2000
			AU 4120899	A	01-11-1999
			AU 4481699	A	01-11-1999
			WO 9952515	A	21-10-1999
			AU 4615599	A	10-01-2000
			WO 9966875	A	29-12-1999
			AU 5158099	A	07-02-2000
			WO 0004031	A	27-01-2000
			AU 5034899	A	07-02-2000
			WO 0003699	A	27-01-2000
			DE 19923567	A	06-04-2000
			WO 0017233	A	30-03-2000
DE 29800547	U	08-04-1999	DE 19752700	A	02-06-1999
			JP 11169186	A	29-06-1999
WO 9953071	A	21-10-1999	AU 3169999	A	01-11-1999
WO 0008169	A	17-02-2000	DE 19835219	A	10-02-2000
			DE 19845216	A	06-04-2000
			DE 19845231	A	06-04-2000
			DE 19845224	A	06-04-2000
			AU 5415799	A	28-02-2000

## A. KLASSIFIZIERUNG DES ANMELDUNGSGEGENSTANDES

IPK 7 C12N15/53 C12N15/54 C12N15/82 C12N9/10 C12N9/04  
C12Q1/02 A01H5/00

Nach der internationalen Patentklassifikation (IPK) oder nach der nationalen Klassifikation und der IPK

## B. RESEARCHIERTE GEBIETE

Recherchierte Mindestprüfstoff (Klassifikationssystem und Klassifikationssymbole)

IPK 7 C12N A01H

Recherchierte aber nicht zum Mindestprüfstoff gehörende Veröffentlichungen, soweit diese unter die recherchierten Gebiete fallen

Während der internationalen Recherche konsultierte elektronische Datenbank (Name der Datenbank und evtl. verwendete Suchbegriffe)

## C. ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
X	<p>LANGE B M ET AL: "A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway."</p> <p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 MAR 3) 95 (5) 2100-4. , XP002116672</p> <p>in der Anmeldung erwähnt</p>	1,2,9, 13,17,18
Y	<p>siehe insbesondere den letzten Absatz</p>	20,21
X	<p>MANDEL A. ET AL.: "CLA1, a novel gene required for chloroplast development, is highly conserved in evolution"</p> <p>PLANT JOURNAL, Bd. 9, Nr. 5, 1996, Seiten 649-658, XP002122907</p> <p>das ganze Dokument</p>	22

-/-

☒ Weitere Veröffentlichungen sind der Fortsetzung von Feld C zu entnehmen

☒ Siehe Anhang Patentfamilie

\* Besondere Kategorien von angegebenen Veröffentlichungen :

"A" Veröffentlichung, die den allgemeinen Stand der Technik definiert, aber nicht als besonders bedeutsam anzusehen ist

"E" älteres Dokument, das jedoch erst am oder nach dem internationalen Anmeldedatum veröffentlicht worden ist

"L" Veröffentlichung, die geeignet ist, einen Prioritätsanspruch zweifelhaft erscheinen zu lassen, oder durch die das Veröffentlichungsdatum einer anderen im Recherchenbericht genannten Veröffentlichung belegt werden soll oder die aus einem anderen besonderen Grund angegeben ist (wie ausgeführt)

"O" Veröffentlichung, die sich auf eine mündliche Offenbarung, eine Benutzung, eine Ausstellung oder andere Maßnahmen bezieht

"P" Veröffentlichung, die vor dem internationalen Anmeldedatum, aber nach dem beanspruchten Prioritätsdatum veröffentlicht worden ist

"1" Spätere Veröffentlichung, die nach dem internationalen Anmeldedatum oder dem Prioritätsdatum veröffentlicht worden ist und mit der Anmeldung nicht kollidiert, sondern nur zum Verständnis des der Erfindung zugrundeliegenden Prinzips oder der ihr zugrundeliegenden Theorie angegeben ist

"X" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann allein aufgrund dieser Veröffentlichung nicht als neu oder auf erfindertlicher Tätigkeit beruhend betrachtet werden

"Y" Veröffentlichung von besonderer Bedeutung; die beanspruchte Erfindung kann nicht als auf erfindertlicher Tätigkeit beruhend betrachtet werden, wenn die Veröffentlichung mit einer oder mehreren anderen Veröffentlichungen dieser Kategorie in Verbindung gebracht wird und diese Verbindung für einen Fachmann naheliegend ist

"Z" Veröffentlichung, die Mitglied derselben Patentfamilie ist

Datum des Abschlusses der internationalen Recherche

17. November 1999

Absenddatum des internationalen Recherchenberichts

03/12/1999

Name und Postanschrift der internationalen Recherchenbehörde  
Europäisches Patentamt, P.B. 5818 Patentaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax: (+31-70) 340-3016

Bevollmächtigter Beauftragter

Kania, T

C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN		
Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
Y	EP 0 723 017 A (BASF AG) 24. Juli 1996 (1996-07-24) Seite 3, Zeile 35-54	20,21
A	WO 97 27285 A (UNIV ARIZONA) 31. Juli 1997 (1997-07-31) in der Anmeldung erwähnt das ganze Dokument	1-22
A	WO 98 06862 A (SHEWMAKER CHRISTINE K ;CALGENE INC (US)) 19. Februar 1998 (1998-02-19) das ganze Dokument	1-22
A	LOIS L M ET AL: "Cloning and characterization of a gene from Escherichia coli encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxyxylulose 5-phosphate, a common precursor for isoprenoid, thiamin and pyridoxol biosynthesis." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1998 MAR 3) 95 (5) 2105-10. , XP002116673 das ganze Dokument	1-22
A	SPRENGER G A ET AL: "Identification of a thiamin-dependent synthase in Escherichia coli required for the formation of the 1-deoxy-D- xylulose 5-phosphate precursor to isoprenoids, thiamin, and pyridoxol." PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA, (1997 NOV 25) 94 (24) 12857-62. , XP002116674 in der Anmeldung erwähnt das ganze Dokument	1-22
A	KELLER ET AL: "metabolic compartmentation of plastid prenillipid biosynthesis - evidence for the involvement of a multifunctional geranylgeranyl reductase" EUROPEAN JOURNAL OF BIOCHEMISTRY, DE, BERLIN, Bd. 251, Nr. 1/02, Seite 413-417-417 XP002100518 ISSN: 0014-2956 in der Anmeldung erwähnt das ganze Dokument	1-22
P,X	WO 99 11757 A (MCCASKILL DAVID G ;LANGE BERND M (US); UNIV WASHINGTON (US); WILDU) 11. März 1999 (1999-03-11) siehe insbesondere S.14 Z.29 bis S.15 Z.21	1,2,9, 13,17-22
	-/-	

## C.(Fortsetzung) ALS WESENTLICH ANGESEHENE UNTERLAGEN

Kategorie*	Bezeichnung der Veröffentlichung, soweit erforderlich unter Angabe der in Betracht kommenden Teile	Betr. Anspruch Nr.
P,X	DE 197 52 700 A (HOECHST SCHERING AGREVO GMBH) 2. Juni 1999 (1999-06-02) siehe insbesondere S.6 Z.20 ff.; Beispiel 6	20,21
E	WO 99 52938 A (HASSAN JOMAA) 21. Oktober 1999 (1999-10-21) das ganze Dokument	18-22

Im Recherchenbericht angeführtes Patentedokument		Datum der Veröffentlichung	Mitglied(er) der Patentfamilie	Datum der Veröffentlichung
EP 0723017	A	24-07-1996	DE 19501906 A	25-07-1996
			CA 2167768 A	24-07-1996
			US 5912169 A	15-06-1999
			US 5925535 A	20-07-1999
WO 9727285	A	31-07-1997	AU 1845397 A	20-08-1997
			EP 0877793 A	18-11-1998
			JP 11510708 T	21-09-1999
WO 9806862	A	19-02-1998	AU 4058497 A	06-03-1998
			CN 1227609 A	01-09-1999
			EP 0925366 A	30-06-1999
WO 9911757	A	11-03-1999	AU 8925898 A	22-03-1999
DE 19752700	A	02-06-1999	DE 29800547 U	08-04-1999
			JP 11169186 A	29-06-1999
WO 9952938	A	21-10-1999	DE 19825585 A	21-10-1999
			WO 9952515 A	21-10-1999

## PATENT COOPERATION TREATY

PCT

From the INTERNATIONAL BUREAU

NOTICE INFORMING THE APPLICANT OF THE  
COMMUNICATION OF THE INTERNATIONAL  
APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:

BOULT WADE TENNANT  
Verulam Gardens  
70 Gray's Inn Road  
London WC1X 8BT  
ROYAUME-UNI

Records

L.D. on Comp.

In Diary

Date of mailing (day/month/year) 03 August 2000 (03.08.00)		PAID 2/00	
Applicant's or agent's file reference SCB/52068001		IMPORTANT NOTICE	
International application No. PCT/GB00/00263	International filing date (day/month/year) 28 January 2000 (28.01.00)	Priority date (day/month/year) 28 January 1999 (28.01.99)	
Applicant ROYAL HOLLOWAY AND BEDFORD NEW COLLEGE et al			

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:

AU,JP,KP,KR,US

In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:

AE,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,CA,CH,CN,CR,CU,CZ,DE,DK,DM,EA,EE,EP,ES,FI,GB,GD,  
GE,GH,GM,HR,HU,ID,IL,IN,IS,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,NO,  
NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,ZA,ZW

The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 49.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on 03 August 2000 (03.08.00) under No. WO 00/44912

## REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a demand for international preliminary examination must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 19-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

## REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the national phase, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

RECEIVED

14 AUG 2000

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

Continuation of Form PCT/IB/308

**NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF  
THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES**

<b>Date of mailing (day/month/year)</b> 03 August 2000 (03.08.00)	<b>IMPORTANT NOTICE</b>
<b>Applicant's or agent's file reference</b> SCB/52068001	<b>International application No.</b> PCT/GB00/00263
<p>The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.</p>	



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/54, 9/10, 1/21, A01H 5/00,</b> <b>C12N 15/82, C12Q 1/48 // (C12N 1/21,</b> <b>C12R 1:19)</b>	<b>A1</b>	<b>(11) International Publication Number: WO 00/44912</b>  <b>(43) International Publication Date: 3 August 2000 (03.08.00)</b>
<b>(21) International Application Number: PCT/GB00/00263</b>  <b>(22) International Filing Date: 28 January 2000 (28.01.00)</b>  <b>(30) Priority Data:</b> 9901902.8                      28 January 1999 (28.01.99)                      GB  <b>(71) Applicant (for all designated States except US):</b> ROYAL HOLLOWAY AND BEDFORD NEW COLLEGE [GB/GB]; University of London, Egham, Surrey TW20 0EX (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> BRAMLEY, Peter, Michael [GB/GB]; Royal Holloway University of London, School of Biological Sciences, Department of Biochemistry, Egham, Surrey TW20 0EX (GB). HARKER, Mark [GB/GB]; 10 Langwood Close, Eaton Sorcon, St. Neots PE19 3QN (GB).  <b>(74) Agent:</b> BOULT WADE TENNANT; Verulam Gardens, 70 Gray's Inn Road, London WC1X 8BT (GB).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the</i> <i>claims and to be republished in the event of the receipt of</i> <i>amendments.</i>
<b>(54) Title: MANIPULATING ISOPRENOID EXPRESSION</b>  <b>(57) Abstract</b>  <p>There is disclosed a method of manipulating isoprenoid expression in a cell or organism having a mevalonate independent isopentyl diphosphate synthesising pathway, which method comprises altering the activity of the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXPS), or a functional equivalent, derivative or bioprecursor thereof. Also disclosed is a transgenic cell, tissue or organism having a mevalonate independent IPP biosynthetic pathway which cell, tissue or organism comprises at least one transgene capable of expressing DXPS or a functional equivalent, derivative or bioprecursor thereof.</p>		

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### MANIPULATING ISOPRENOID EXPRESSION

The present invention is concerned with manipulating or altering isoprenoid expression in a cell or  
5 organism which biosynthesises isopentenyl diphosphate (IPP), which is the universal precursor of all isoprenoids in nature, via a mevalonate independent pathway.

10 Isoprenoids constitute the largest class of natural products occurring in nature, with over 29,000 individual compounds identified to date [1]. Chemically, they are extremely diverse in their structure and complexity. The fundamental biological  
15 functions performed by isoprenoids ensure they are essential for the normal growth and developmental processes in all living organisms. These include functioning as eukaryotic membrane stabilisers (sterols), plant hormones (gibberellins and abscisic  
20 acid), providing pigments for photosynthesis (carotenoids and phytol side chain of chlorophyll), and as carriers for electron transport (menaquinone, plastoquinone and ubiquinone).

25 All isoprenoids are synthesised via a common metabolic precursor, isopentenyl diphosphate (IPP; C<sub>5</sub>). Until recently, the biosynthesis of IPP was generally assumed to proceed exclusively from acetyl-CoA via the classical mevalonate pathway (Fig. 1) [2]. The enzyme  
30 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGR) catalyses the conversion of hydroxymethylglutaryl-CoA to mevalonate, a key reaction of the mevalonate-dependant IPP biosynthetic pathway. Recent  
35 studies have demonstrated that mevalonate is not the biosynthetic precursor of IPP in all living organisms

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[3,4]. The existence of an alternative, mevalonate-independent pathway for IPP formation was identified initially in several species of eubacteria [4,5] and a green alga [6]. The pathway was subsequently shown to be operational in the plastids of higher plants [7-10]. The first reaction in the non-mevalonate pathway is the transketolase-type condensation reaction of pyruvate and D-glyceraldehyde-3-phosphate to yield 1-deoxy-D-xylulose-5-phosphate (DXP) (Fig. 1). This reaction is catalysed by the enzyme 1-deoxy-D-xylulose-5-phosphate synthase. The second reaction in the pathway is the conversion of DXP to 2-C-methyl-D-erythritol-4-phosphate (MEP). The reactions which convert MEP to IPP have yet to be characterised.

The cloning and characterisation of the DXP synthase (*dxps*) gene has been described for a number of organisms including *Escherichia coli* [11,12] and higher plants [13-15]. The *CLA1* gene product from *Arabidopsis thaliana* associated with chloroplast development [16], for example, has been shown to exhibit *DXPS* activity [11]. Recently, a gene responsible for the reduction of DXP to 2-C-methyl-D-erythritol-4-phosphate, the proposed next step in the non-mevalonate pathway has been cloned from *E. coli* [17].

The present inventors have surprisingly found that the first reaction in the mevalonate-independent IPP biosynthetic pathway is highly influential in controlling the levels of isoprenoids which can be formed in a cell or organism within which the mevalonate independent IPP biosynthetic pathway is present. The enzyme *DXPS* or functional equivalents

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thereof, has been identified by the present inventors as a rate-limiting step in isoprenoid biosynthesis and that DXPS activity plays an important role in channelling the carbon resources of the cell into the isoprenoid biosynthetic pathway.

Therefore, according to a first aspect of the present invention there is provided a method of manipulating isoprenoid expression in a cell possessing a mevalonate independent isopentenyl diphosphate synthesising pathway, which method comprises altering the activity of the enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXPS), or a functional equivalent thereof. Thus, advantageously, the rate-limiting effect conferred by DXPS activity on the IPP biosynthetic pathway can be utilised to manipulate the resultant levels of isoprenoids in a cell by altering the activity or expression of DXPS.

Preferably, the levels of isoprenoids in a cell can be enhanced by increasing the activity or expression of the DXPS. Likewise reduced levels of isoprenoids can be achieved by reducing or inhibiting activity or expression of DXPS in a cell or organism. Increasing the DXPS activity may be achieved by, for example, transforming the cell which may itself be part of a cell line or an organism, with an expression vector comprising a nucleic acid molecule encoding DXPS, which may advantageously be operably linked to a reporter molecule, such as used in the GUS assay which is known in the art. Preferably, the vector comprises any of the vectors designated pBSDXPS or pSYDXPS, illustrated in Figure 2.

35

An alternative method for altering expression may comprise utilising a technique known as Enforced Evolution, or DNA Shuffling see Patten et al. Current Opinion in Biotechnology, 1997, Vol. 8, No. 6, pp 724-733, Crameri et al., Nature 1998, Vol. 391, No. 6664, pp 288-291 and Harayama S, Trends in Biotechnology, 1998, Vol. 16, No. 2, pp 76-82. According to this method improvements in enzyme activity can be achieved by reassembling DNA segments into a full length gene by homologous or site specific combination. Before the assembly, the segments are often subjected to random mutagenesis by error prone PCR, random nucleotide insertion, or other such methods. The genes can be expressed in suitable microbial hosts leading to the production of functional polypeptides, such as DXPS.

The nucleic acid encoding the DXPS may be endogenous to the cell or organism into which it will be transformed or, alternatively, may be exogenous. In one embodiment of the invention, the method may also comprise transforming the cell or organism with a vector comprising one or more nucleic acid sequences suitable for producing a desired isoprenoid. This aspect of the invention is particularly advantageous because it allows isoprenoids to be produced in a cell or organism independent of the source of the isoprenoid which may be derived from cells or organisms which do not possess the mevalonate independent IPP biosynthesising pathway. Similarly, enhanced levels of an isoprenoid can be produced in cells or organisms having the mevalonate independent IPP biosynthetic pathway.

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Therefore, in the example where the cell is *E. coli* it is possible to engineer production of an isoprenoid which is exogenous to the *E. coli* bacterium, which isoprenoid may be, for example, any of the carotenoids of plants, such as, lycopene or even an isoprenoid of human origin.

Carotenoids are yellow-orange-red lipid based pigments found in nature. They have been found to be useful in a variety of applications, for example, as supplements, and particularly vitamin supplements, as vegetable oil based food products and food ingredients, as feed additives in animal feeds and as colorants. Phytoene has been found to be useful in treating skin disorders whilst lycopene and  $\alpha$  and  $\beta$  carotene consumption have been implicated as having preventative effects against certain kinds of cancers. Therefore, it is a highly advantageous aspect of the invention that increased production of such compounds can be achieved and which compounds can confer considerable health care benefits. Once the desired carotenoid or other isoprenoid has been produced in *E. coli*, or other suitable organism as defined above, it can be isolated using standard biocengineering techniques.

Increases in concentrations of any desired isoprenoid may be achieved, in a cell or alternatively an organism which possesses the IPP biosynthetic mevalonate independent pathway. For example, crops can be engineered using the method of the invention to produce increased levels of an isoprenoid which confers nutritional benefits to humans following consumption of the plant, such as, for example, vitamin E and lycopene.

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Therefore, there is also provided by a further aspect of the invention a cell or organism having a mevalonate independent IPP biosynthetic pathway and which has been transformed or transfected with an expression vector comprising a nucleic acid molecule encoding DXPS or a functional equivalent or bioprecursor thereof. As described above, the vector may also include one or more further nucleic acid sequences suitable for producing a desired isoprenoid, or alternatively the one or more nucleic acid sequences may be included in a separate vector, operably linked to suitable expression control sequences. In a particularly preferred embodiment the cell or organism comprises a plant.

An expression vector according to the invention includes a vector having a nucleic acid sequence operably linked to regulatory sequences, such as promoter regions, that are capable of effecting expression of said DNA fragments. The term "operably linked" refers to a juxta position wherein the components described are in a relationship permitting them to function in their intended manner. Such vectors may be transformed into a suitable host cell or organism to produce a desired protein, such as DXPS or an isoprenoid according to the method of the invention. Thus, in a further aspect, the invention provides a process for producing a desired isoprenoid which comprises cultivating a host cell, transformed or transfected with an expression vector as described above under conditions to provide for expression by the vector of DXPS or a functional equivalent thereof or suitable polypeptides for producing a desired isoprenoid and optionally recovering the expressed polypeptides.

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The vectors may be, for example, plasmid, virus or phage vectors provided with an origin of replication, optionally a promoter for the expression of said nucleotide and optionally a regulator of the promoter.

5 The vectors may contain one or more selectable markers, such as, for example, ampicillin resistance.

Regulatory elements required for expression include promoter sequences to bind RNA polymerase and

10 transcription initiation sequences for ribosome binding. For example, a bacterial expression vector may include a promoter such as the lac promoter and for transcription initiation in the Shine-Dalgarno sequence and the start codon AUG. Similarly, a

15 eukaryotic expression vector may include a heterologous or homologous promoter for RNA polymerase II, a downstream polyadenylation signal, the start codon AUG, and a termination codon for detachment of the ribosome. Such vectors may be obtained

20 commercially or assembled from the sequences described by methods well known in the art.

By combining the nucleic acid sequences encoding said DXPS and optionally the one or more sequences suitable

25 for producing an isoprenoid with tissue specific promoters, enhanced isoprenoid levels in specified tissues of plants can be achieved. For example, by utilising a seed specific promoter or other

30 transcriptional initiation region, elevated levels of carotenoids in seeds may be achieved. The seed can then be harvested and which provides a reservoir for the isoprenoid or carotenoid of interest.

Generally, the nucleic acid molecule encoding said

35 DXPS which is included in the vector used in

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accordance with the method of the invention, will be transformed into a plant cell so that the DXPS molecule is directed to the plastids of the plant. Accordingly, where the vector is not inserted directly into the plastid of the plant, the vector will further comprise a nucleic acid sequence operably linked to said DXPS or said one or more isoprenoid producing nucleic acid sequences and which further sequence will encode a transit peptide to direct expression of the DXPS or isoprenoid into the plastid. Native or heterologous transit peptides may be utilised in this embodiment of the invention.

As aforesaid, the mevalonate independent IPP biosynthetic pathway is not present in any higher animals, particularly humans. Therefore, the inhibition of the reaction catalysed by DXPS provides a unique target site to selectively inhibit or alleviate bacterial associated infections by altering the expression level of or inhibiting function or activity of DXPS.

One method of inhibiting or preventing expression of DXPS utilises antisense technology. Antisense technology can be used to control gene expression through helix formation of antisense DNA or RNA, both of which methods are based on polynucleotide binding to DNA or RNA. For example, the 5'-coding region of a native DNA sequence coding for DXPS according to the invention may be used to design an antisense RNA nucleotide of from 10 to 50 base pairs in length. A DNA oligonucleotide is designed to be complementary to a region of the gene involved in transcription (triple-helix - see Lee et al, Nucl. Acids. Res., 6:3073 (1978); Cooney et al., Science, 241:456 (1988);

and Derman et al., Science 251:1360 (1991), in which case expression of the antisense RNA oligonucleotide allows hybridisation to the mRNA *in vivo* and blocks translation of an mRNA molecule into DXPS.

5

Alternatively, compounds can be screened for their ability to inhibit the catalytic activity or expression of DXPS in the mevalonate - independent IPP biosynthetic pathway. According to a further aspect of the invention, therefore, there is also provided a method of identifying a compound which modulates isoprenoid production or expression which method comprises contacting said compound to be tested with a molecule from the mevalonate independent IPP biosynthetic pathway and which molecule undergoes a reaction in the presence of an appropriate reactant catalysed by DXPS, in the presence of DXPS and monitoring the level of product produced when compared to the same reaction in the absence of the compound to be tested. Preferably, the molecules which are reacted are pyruvate and glyceraldehyde-3-phosphate, and which undergo a condensation reaction in the presence of DXPS, to yield 1-deoxy-D-xylulose-5-phosphate (DXP) as illustrated in Figure 1.

25

Any compounds identified as preventing expression or activity of the DXPS enzyme according to the invention may advantageously be particularly useful as selective toxicity agents to destroy, for example, bacterial or plant cells which possess the mevalonate independent IPP biosynthetic pathway. These compounds therefore can be particularly useful as medicaments or herbicides, or alternatively in the preparation of a medicament for treating bacterial associated diseases.

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A further aspect of the invention therefore also comprises a pharmaceutical composition comprising a compound identified as an inhibitor of expression or activity of DXPS or a functional equivalent or bioprecursor thereof, together with a pharmaceutically acceptable carrier, diluent or excipient thereof. Also provided by the invention is a herbicidal composition comprising said compound identified as an inhibitor of expression or activity of DXPS function.

10 An even further aspect of the invention comprises a transgenic cell, tissue or organism having a mevalonate independent IPP biosynthetic pathway, which comprises a transgene capable of expressing at least one additional DXPS molecule according to the invention. The transgenic cell, tissue or organism may also comprise a transgene having one or more nucleic acid sequences capable of producing a desired isoprenoid. Preferably, the transgenic cell comprises a plant and even more preferably tomato plants.

The term "transgene capable of expression" as used herein means a suitable nucleic acid sequence(s) which leads to expression of DXPS or proteins having the same function and/or activity and/or encoding proteins capable of producing a desired isoprenoid. The transgene, may include, for example, isolated genomic nucleic acid or synthetic nucleic acid, including DNA integrated into the genome. Preferably, the transgene comprises the nucleic acid sequence(s) encoding the DXPS enzyme or said isoprenoid as described herein, or a functional fragment of said nucleic acid. A functional fragment of said nucleic acid should be taken to mean a fragment of the gene comprising said nucleic acid(s) coding for the DXPS enzyme or said

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isoprenoid or a functional equivalent, derivative or a non-functional derivative such as a dominant negative mutant, or bioprecursor thereof. For example, it would be readily apparent to persons skilled in the art that nucleotide substitutions or deletions may be made using routine techniques, which do not affect the protein sequence and subsequent functioning of the DXPS enzyme and/or isoprenoid producing proteins encoded by said nucleic acid(s).

The DXPS enzyme expressed or the isoprenoid produced by said transgenic cell, tissue or organism or a functional equivalent or bioprecursor of said protein also forms part of the present invention.

The recombinant DNA molecules or vectors of the invention can be introduced into a plant cell in a number of recognised ways in the art and it will be appreciated that the choice of method used might depend on the type of plant, i.e. monocot or dicot, targeted for transformation. Suitable methods of transforming plant cells include microinjection (Crossway *et al.* (1986) *BioTechniques* 4:320-334), electroporation (Riggs *et al.* (1986) *Proc. Natl. Acad. Sci. USA* 83:5602-5606, *Agrobacterium* mediated transformation (Hinchee *et al.* (1988) *Biotechnology* 6:915-921) and ballistic particle acceleration (see, for example, Sanford *et al.*, U.S. Patent 4,945,050; and McCabe *et al.* (1988) *Biotechnology* 6:923-926).

Alternatively, in the case of an organism, such as a plant, a plastid can be transformed directly. Stable transformation of chloroplasts has been reported in higher plants, see, for example, SVAB *et al.* (1990) *Proc. Natl. Acad. Sci. USA* 87:8526-8530; SVAB & Maliga

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(1993) Proc. Natl. Acad. Sci. USA 90:913-917; Staub & Maliga (1993) Embo J. 12:601-606. The method relies on particle gun delivery of DNA containing a selectable marker and targeting of the DNA to the plastid genome through homologous recombination. In such methods, plastid gene expression can be accomplished by use of a plastid gene promoter or by trans-activation of a silent plastid-borne transgene positioned for expression from a selective promoter sequence such as that recognised by T7 RNA polymerase. The silent plastid gene is activated by expression of the specific RNA polymerase from a nuclear expression construct and targeting of the polymerase to the plastid by use of a transit peptide. Tissue-specific expression may be obtained in such a method by use of a nuclear-encoded and plastid-directed specific RNA polymerase expressed from a suitable plant tissue specific promoter. Such a system has been reported in McBride et al. (1994) Proc. Natl. Acad. Sci., USA 91:7301-7305.

The cells which have been transformed may be grown into plants in accordance with conventional methods known in the art. See, for example, McCormick et al., Plant Cell Reports (1986), 5:81-84. These plants may then be grown, and either pollinated with the same transformed strain or different strains, and the resulting hybrid having the desired phenotypic characteristic identified. Two or more generations may be grown to ensure that the subject phenotypic characteristic is stably maintained and inherited and then seeds harvested to ensure the desired phenotype or other property has been achieved.

A host cell of any plant variety may be employed.

Plant species which provide seeds of interest are particularly useful. For the most part, plants will be chosen where the seed is produced in high amounts, a seed-specific product of interest is involved, or  
5 the seed or a seed part is edible. Seeds of interest include the oil seeds, such as oilseed Brassica seeds, cotton seeds, soybean, safflower, sunflower, coconut, palm, and the like; grain seeds, e.g. wheat, barley, oats amaranth, flax, rye, triticale, rice, corn, etc.;  
10 other edible seeds or seeds with edible parts including pumpkin, squash, sesame, poppy, grape, mung beans, peanut peas, beans, radish, alfalfa, cocoa, coffee, tree nuts such as walnuts, almonds, pecans, chick-peas etc.

15 The invention may be more clearly understood from the following exemplary embodiment described with reference to the accompanying drawings wherein:

20 Figure 1: is an illustration of the mevalonate-dependant (A) and independent (B) pathways for IPP biosynthesis. Proposed reactions for the biosynthesis of  
25 1-deoxy-D-xylulose-5-phosphate from pyruvate and glyceraldehyde-3-phosphate, catalysed by *DXPS* is as shown inside the box.

30 Figure 2: is an illustration of structure of plasmids pBSDXPS and pSYDXPS.

Figure 3: is an illustration of an amino acid sequence alignment of *DXP* synthases  
35 used in the present invention,

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5 Synechocystis sp. 6803 (S.s) (GenBank  
D90903), B. subtilis (B.s) (GenBank  
D84432) and E. coli (E.c) (GenBank  
AF035440). The consensus line (consen)  
shows residues conserved in all three  
sequences (upper case letters) or  
residues which are identical in two  
sequences and replaced by an equivalent  
amino acid in the third sequence (+).  
10 The conserved histidine domain  
putatively involved in proton transfer  
is over lined and numbered 1. The  
second over lined domain (2) denotes  
the consensus thiamin pyrophosphate  
15 (TPP)-binding motif.

Figure 4: is a graphic representation of lycopene  
accumulation in recombinant E. coli  
cultures expressing vector only ( $\square$ ),  
20 B. subtilis DXPS ( $\bullet$ ) and Synechocystis  
sp. 6803 DXPS ( $\Delta$ ). (Data are means  $\pm$   
S.E.M. from three independent  
determinations.)

25 Figure 5: is an illustration of lycopene (open  
columns) and UQ-8 (shaded columns)  
content of E. coli control cultures  
(vector only) or expressing exogenous  
B. subtilis dxps (B. subtilis),  
30 Synechocystis sp. 6803 dxps (sp. 6803)  
or A. thaliana hmgr1 (HMGR1) genes.  
(Data are means ( S.E.M. from three  
independent determinations.)

35 Figure 6: is a diagrammatic illustration of

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vector pVB6\_TSEC\_LML.

Figure 7: is a diagrammatic representation of  
plasmid pVB6\_35S\_TSEC-LML.

5

Figure 8: is an illustration of the amino acid  
sequence of *E.coli* DXPS.

Figure 9: is an illustration of the transit  
peptide used in tomato plants.

10

#### EXAMPLE 1

#### 15 **Materials and methods**

Bacterial strains, plasmids, and culture conditions.

*E. coli* strain XL1-Blue (Stratagene) was used for gene  
cloning and expression of plasmids. *E. coli* was grown  
20 in Luria Broth media [18] at 37°C on a rotary shaker  
at 250 rpm (unless otherwise stated). Ampicillin (100  
µg/ml), chloramphenicol (50 µg/ml) and 1.0 mM  
isopropyl-β-D-thiogalactoside (IPTG) (all purchased  
25 from Sigma) were added as required. Plasmid  
pBluescript (Stratagene) was used as a vector for both  
cloning and expression studies. *Synechocystis* sp. PCC  
6803 was obtained from the Institute Pasteur (Paris)  
and grown in BG11 medium [19] supplemented with 0.5%  
30 glucose at 30°C and 2,000 lux. *Bacillus subtilis*  
strain PY79 DNA was a kind gift from P. Wakeley (Royal  
Holloway, University of London). The construction of  
plasmid pACCRT-EIB, which expresses the *E. uredovora*  
crtE, crtB and crtI genes necessary for lycopene  
35 biosynthesis in *E. coli* cells into which it has been

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introduced, has been described previously [20]. The plasmid used for the expression of HMGR1 cloned into pBluescript (pHMGR1) has also been described elsewhere [21].

5

#### Recombinant DNA techniques

All recombinant DNA techniques were performed by standard methods [22] or according to suppliers  
10 instructions. Genomic DNA was extracted from all organisms using the Qiagen Genomic-tip 20/G kit.

#### Cloning of dxps genes

15 Based on the nucleotide sequence of ORF sll1945 from the genome database for *Synechocystis* sp. PCC 6803 [23], primers were designed to clone the putative dxps gene by polymerase chain reaction (PCR). The forward primer 5'-GTCCAATCCACCATGCACATCAG-3' overlaps  
20 the beginning of the coding sequence. The reverse primer 5'-CCCTCGACAAATGCAAAATGTATC-3' lies outside the stop codon of the gene. A PCR (25 cycles) using Pfu DNA polymerase (Stratagene) produced a DNA fragment of the expected size (~1.9 kb). Subsequent sequencing of  
25 the fragment confirmed the product to be the ORF sll1945. The *B. subtilis* dxps gene was also cloned by PCR using primers designed to amplify the gene encoding the product YqiE, identified in the *Bacillus subtilis* genome database [24]. The forward primer  
30 5'-GATCCGCTATGGATCTT TTATC-3' contains a modified base substitution at the predicted start codon (underlined) for improved expression in *E. coli*. The reverse primer 5'-ATCTAATCGTTCTTTCTTTGAC-3' lies outside the stop codon of the dxps gene. After PCR (25 cycles) a  
35 DNA product of the expected size (~1.9 kb) was

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obtained, and when sequenced proved to be identical to the gene encoding the product YqiE. The PCR products from both reactions were treated with Taq DNA polymerase (GibcoBRL) at 72°C for 10 min to synthesise blunt ended fragments. The fragments were then cloned into the EcoRV site of the pBluescript vector (Stratagene) using T4 DNA ligase (Fermentas) (Fig. 2).

#### *In vitro* DXP synthase assay

*E. coli* XL1-blue cells, transformed with the appropriate plasmid, were grown at 37°C in Luria Broth medium with appropriate antibiotics to an OD<sub>620 nm</sub> of 0.6, and induced by the addition of 1.0 mM IPTG at 28°C for two hours. Bacteria were harvested by centrifugation (6,000g for 10 min) and washed in buffer A (100 mM Tris-HCl (pH 7.5), 1 mM dithioreitol, 0.3 M sucrose). Cells were resuspended to their original volume in buffer B (100 mM Tris (pH 8.0), 1 mM dithioreitol, 0.1 mM phenylmethanesulphonyl fluoride, 1 µ/ml pepstatin, 1 µg/ml leupeptin, 1 mg/ml lysozyme). The cells were then incubated at 30°C for 15 min with gentle agitation, and then disrupted by brief sonication at 4°C. The supernatant was recovered and the protein concentration determined using the Bradford assay [25].

An aliquot of the supernatant (100 µl) was transferred to an Eppendorf tube along with 100 µl of assay buffer containing 100 mM Tris (pH 8.0), 3 mM ATP, 3 mM Mn<sup>2+</sup>, 3 mM Mg<sup>2+</sup>, 1 mM KF, 1 mM thiamine diphosphate, (0.1%) Tween 60, 0.6 mM mDL-glyceraldehyde-3-phosphate, 30 µM [2-<sup>14</sup>C]pyruvate (0.5 µCi). The mixture was incubated for 2 hours at

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30°C with gentle agitation. The reaction was stopped by heating the mixture at 80°C for 3 min. After centrifugation at 13,000 g for 5 min, the supernatant was transferred to a clean tube and evaporated to dryness. The residue was resuspended in methanol (50  $\mu$ l) and loaded onto a TLC plate (silica gel 60). Chromatograms were developed in *n*-propyl alcohol/ethyl acetate/H<sub>2</sub>O (6:1:3 v/v/v).

Enzyme assays were performed with extracts of induced cells expressing either *Synechocystis* sp. PCC 6803 or *B. subtilis* DXPS, as opposed to control assays in which cells contained only the pBluescript vector without insert. TLC analysis of assays expressing one of the dxps clones exhibited a major band ( $R_f$  0.14 ) assumed to be DXP which was not observed in the controls. Quantification of <sup>14</sup>C-labelled DXP was achieved by isolation of the reaction product on TLC. The DXP band was scraped off the plate, eluted from the silica using methanol and quantified by liquid-scintillation counting. Enzymatic dephosphorylation of the assay products resulted in the formation of 1-deoxy-D-xylulose (DX), when analysed on TLC ( $R_f$  0.50). When non-radioactive pyruvate was used in the assay, the DXP ( $R_f$  0.12 stained purple) and DX ( $R_f$  0.47 stained blue) were identified by staining with *p*-anisaldehyde/sulphuric acid (3:1). The DXP co-chromatographed with authentic, chemically synthesised DXP which stained purple also. The reaction substrates pyruvate ( $R_f$  0.36 stained yellow), DL-glyceraldehyde-3-phosphate ( $R_f$  0.15 stained orange) and D-glyceraldehyde ( $R_f$  0.74 stained orange) were also observable using this TLC system. In reactions where the assay products were dephosphorylated no DXP was observed on TLC only DX.

Quantification of lycopene and ubiquinone QB-8 in *E. coli*

Bacterial growth was determined from the OD<sub>620 nm</sub>. Dry  
5 cell weight was calculated from known volumes of  
culture harvested by centrifugation at 13,000 g for 5  
min, washed once with water and recentrifuged. The  
cells were lyophilised overnight and the weight of the  
dried cell pellet determined. The lycopene content of  
10 the cells was determined by harvesting aliquots of *E. coli*  
cells by centrifugation at 13,000 g for 5 min and  
washing once in water followed by recentrifuging. The  
cells were resuspended in acetone (200  $\mu$ l) and  
incubated at 68°C for 5 min in the dark. The samples  
15 were centrifuged again 13,000 g for 10 min and the  
acetone supernatant containing the lycopene was placed  
in a clean tube. The extract was evaporated to  
dryness under a stream of N<sub>2</sub> and stored at -20°C in  
the dark. The lycopene content of the extracts was  
20 determined by visible light absorption spectra using a  
Beckman DU Series 7000 diode array spectrometer.  
Spectra were recorded in acetone using an A<sup>1%</sup><sub>1cm</sub> of 3450  
[26].

25 UQ-8 was extracted from cells based on the methods of  
Yoshida et al. [27]. Cells were collected by  
centrifugation, washed once with water and then  
lyophilised overnight. The dried pellet was extracted  
in *n*-propanol (3 ml) and of *n*-hexane (5 ml) containing  
30 15  $\mu$ g of UQ-10 as an internal standard, by disruption  
of the cells using a pestle and mortar. The solvent  
phase and that obtained by the second extraction from  
the aqueous phase *n*-hexane (3 ml) were combined and  
evaporated to dryness under N<sub>2</sub>. The residue was  
35 resuspended in ethanol and analysed by reversed phase

- 20 -

HPLC as described previously [28]. Peaks were identified by comparing their elution profiles with standards for UQ-7, UQ-9 and UQ-10. A standard of UQ-8 was not available, and the UQ-8 peak was identified by its elution profile relative to those of the other standards [29].

#### Cloning of the dxps genes

The cloning of dxps and the characterisation of the gene product, DXPS, from *E. coli* has recently been reported by two research groups [11,12]. The gene product was shown to exhibit DXP synthase activity, which is considered as the first reaction of the mevalonate-independent pathway for IPP biosynthesis (Fig. 1) [5]. Based on the *E. coli* dxps nucleotide sequence homologs of the gene were identified in the eubacterial genomes of *B. subtilis* and *Synechocystis* sp. PCC 6803. The open reading frame sll1945 in the *Synechocystis* sp. 6803 genome was cloned by PCR, ligated into the vector pBluescript, and designated pSYDXPS (Fig. 2). The gene extends over 1920 bp and contains an open reading frame encoding a polypeptide of 640 amino acids, with a predicted molecular mass of 69 kDa. The dxps homolog in the *B. subtilis* genome was identified as the ORF encoding the product YqiE. It was cloned by PCR, and introduced into pBluescript to generate plasmid pBSDXPS (Fig. 2). The gene extends over 1899 bp and encodes a polypeptide of 633 amino acids with a predicted molecular mass of 70 kDa.

The amino acid sequence of the DXPS proteins of *Synechocystis* sp. 6803 and *B. subtilis* exhibited significant similarity to each other over their entire length (47% identities) and to the *E. coli* DXPS (*B.*

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subtilis (44 % identities) and Synechocystis sp. 6803 (46 % identities)) (Fig. 3). All three polypeptides share two conserved domains; one thought to be involved in thiamin binding [30] and a histidine residue postulated to participate in proton transfer [31], both of which are detailed in Fig. 3. The existence of a thiamin-binding domain in each of the polypeptides explains the cofactor requirement of thiamin for DXPS activity [12]. The high degree of polypeptide sequence identity, particularly the distribution of conserved domains, in all three indicates that they all encode DXPS or a closely related gene product.

#### Quantification of lycopene and UQ-8 in *E. coli* transformants

Cells of *E. coli* transformed with pACCRT-EIB [20] are pigmented pink due to the accumulation of lycopene. *E. coli* cells engineered to produce lycopene, were transformed with either pBSDXPS, pSYDXPS, pHMGR, or pBluescript to act as a control, to monitor the effect on lycopene biosynthesis when exogenous DXPS was expressed in the cells. The *E. coli* were grown in 50 ml cultures at 30°C with induction by IPTG for 48 hours, by which time they had reached the stationary phase of growth. Figure 4 shows the accumulation of lycopene in the cultures during the 48 hour culture period. The graph clearly demonstrates that the *E. coli* cultures expressing exogenous dxps accumulated lycopene at a much greater rate than the control culture. The final lycopene content of the recombinant dxps strains was approximately double that of the control (Fig. 5). A similar increase was also obtained in *E. coli* cells engineered to produce the

colourless carotenoid phytoene (data not shown). Alterations in the endogenous levels of isoprenoids were determined by measuring the ubiquinone content of the cells. In *E. coli*, the major quinones  
5 encountered are ubiquinone (UQ-8) and menaquinone (MK-8) [32]. Ubiquinone is a major component of the aerobic respiratory chain. It is estimated that there are approximately 50 molecules of ubiquinone for each of the oxidation complexes in *E. coli* [33]. By  
10 measuring an end product which is produced in relatively large quantities, it was conjectured that alterations in the rates of biosynthesis could be readily detected. The UQ-8 content of the recombinant dxps strains was 1.5 times greater than the controls  
15 (Fig. 5). Lycopene and UQ-8 levels were measured in *E. coli* transformed with *hmgr1* from *A. thaliana*, to monitor if this caused any alterations in the isoprenoid content of the cells. Expression of the *A. thaliana* *hmgr1* cDNA had no effect of the levels of  
20 lycopene nor UQ-8 in the cells (Fig. 5).

The results show that increased expression of DXPS leads to increased lycopene and UQ-8 levels in the recombinant *E. coli* cells. This indicates that  
25 increasing the rate of DXP synthesis, the initial reaction in the mevalonate-independent pathway for IPP biosynthesis, elevates isoprenoid production. In contrast, expression of *hmgr1* had no effect on isoprenoid biosynthesis, suggesting that mevalonate  
30 dependent IPP biosynthesis has little or no role in IPP synthesis in *E. coli*. Similarity searches of the *E. coli* genome data base for proteins of the mevalonate-dependent IPP biosynthesis pathway failed to identify any possible homologs in the genome  
35 suggesting that this pathway is probably absent in

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this organism.

*In vitro* enzyme activity

5     The increased levels of carotenoids and UQ-8 in *E. coli* expressing exogenous DXPS were hypothesised to be due to increased DXPS enzymatic activity in the cells. This was confirmed by preparing cell homogenates from recombinant *E. coli* strains after induction with IPTG.

10    Reaction products were measured over a two hour period, separated by TLC and quantified by liquid-scintillation counting. The major product obtained from the reaction co-chromatographed with chemically-synthesised DXP. This confirms DXP as the

15    major reaction product in the assay. The putative DXPS function of *B. subtilis* ORF encoding the product YqiE and *Synechocystis* sp. 6803 ORF sll1945 has been established by these results. Table 1 shows the specific activity of DXPS in the recombinant *E. coli*

20    strains. The results show that DXPS activity was increased in *E. coli* expressing endogenous dxps genes. This increase was greatest in homogenates containing the *B. subtilis* DXPS, where a 2.0 fold increase was observed compared to the controls. Homogenates

25    containing the *Synechocystis* sp. 6803 DXPS exhibited a 1.8 fold increase compared to control reactions. Therefore, increased DXPS activity in *E. coli* appears to be responsible for the increased levels of carotenoids and UQ-8 observed in the transgenic

30    strains. The relative increases in carotenoid levels between *E. coli* cultures expressing plasmids pSYNDXSP and pBSDXPS closely resemble the increases observed in the *in vitro* studies. This suggests that there is a direct relationship between DXPS activity and the

35    carotenoid content of the cells. This is not the case

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for UQ-8 where increases in the levels of UQ-8 are more restricted, which could be due to a rate-limiting reaction later in the UQ-8 biosynthesis pathway [34]. The results support the hypothesis that increased DXPS activity in *E. coli* results in increased levels of carotenoids and UQ-8. These data suggest that isoprenoid levels in *E. coli* can be increased by enhancing DXPS activity.

## 10 Transformation Protocols in Tomato Plants

### Triparental mating

Liquid LB medium (5ml) containing rifampicin (100 $\mu$ g/ml) was inoculated with a single *Agrobacterium tumefaciens* colony picked from an LB/rif plate. It was then incubated in a 27°C shaking incubator (225-250rpm) for 48 hours in the dark. Single colonies of Helper strain *E.coli* HB101/pRK2013 (kanamycin resistant) and the donor were also picked and grown up overnight at 37°C in LB liquid medium with appropriate antibiotics. Following the incubation period each bacterial culture was centrifuged at 10,000rpm for 2 minutes. The supernatants were discarded and the pellets resuspended in LB liquid medium. Aliquots of each strain (100 $\mu$ l each) were then mixed and spread with a sterile spreader onto an LB plate with no selection. The plate was inverted and incubated overnight at 27°C in the dark. A loopful of the overnight mating mix was then streaked onto a LB plate containing selective antibiotics (rifampicin, 100 $\mu$ g/ml and kanamycin 50 $\mu$ g/ml). The plate was inverted and incubated for 48-72 hours at 27°C in the dark. Single colonies could then be selected for use in transformation of tomato explants.

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## Seed sterilisation

Ailsa craig variety of tomato seeds were placed into a sterile 50ml Falcon tube. The seeds were washed with 70% ethanol for 30 seconds and the ethanol removed. 1% Virkon was then added and the tube incubated with shaking at 27°C for 20-30 minutes. 1% Virkon was then added and the tube incubated with shaking at 27°C for 20-30 minutes. The seeds were then washed with sterile dH<sub>2</sub>O (~500ml) through a sterile sieve.

## Seed sowing

MS3S medium (125ml) was poured per sterile double Magenta pot (Sigma) and allowed to set.

Five sterile seeds were then sown in each pot and incubated for 5 weeks in a control temperature room (27°C) under 5 cool white light tubes with 16 hours photoperiod and 70% relative humidity.

## Explant preparation

Plates were prepared for explant preparation by the addition of MS3C5ZR medium to petri dishes (25 plates per litre of medium). A sterile 8.5cm filter disc was then placed onto each plate. Plates were wrapped in cling film and stored at room temperature. Explants were taken under aseptic conditions for 5 week old seedlings. 1-1.5cm sections from above cotyledons were cut and all leaves, roots and leaf nodes were removed. The explants were placed on a filter disc on pre-incubation medium (10 per plate as prepared in step 1. The plates were then sealed and stored at 26°C with low light intensity.

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*A. tumefaciens* culture preparation

Several *A. tumefaciens* colonies from triparental mating containing either pVB6\_TSEC-LML or  
5 pVB6\_35S\_TSEC-LML were picked and used to inoculate LB liquid medium (10mls) containing kanamycin (50 $\mu$ g/ml). The culture was incubated in a shaking incubator (225-250rpm) for 24 hours at 27°C. The overnight culture (10mls) was added to LB liquid medium (50mls)  
10 containing kanamycin (50 $\mu$ g/ml). This second culture was then incubated for 24 hours at 27°C in a shaking incubator (225-250rpm).

The *A. tumefaciens* culture (40mls) was then briefly  
15 centrifuged in a bench-top centrifuge (up to 3,000rpm) to remove clumps of growth. The supernatant was then carefully collected into a sterile 50ml Falcon tube. The supernatant was spun at 3,000rpm in a bench-top centrifuge for 10 minutes and the supernatant  
20 discarded. The pellet was resuspended in MS3S (30mls) by vortexing. The culture was diluted to 1/10<sup>th</sup> with MS3S and the optical density (OD) at 550nm measured with MS3S as a blank. The OD was adjusted to 0.1 with MS3S 20-25mls of culture was prepared for every 50  
25 explants transformed.

## Transformation of explants

50 explants were prepared as above (5 plates) and were  
30 transferred into petri dishes and 25ml of *A. tumefaciens* solution per petri dish poured over them. They were then incubated at room temperature for 10 minutes before being transferred to petri dishes containing a double layer of sterile filter paper.  
35 The explants were then transferred to plates

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containing MS3SC5ZR medium (10 per plate). The plates were sealed and then incubated in a control temperature room (27°C) for 48 hours.

## 5 Selection

The explants were transferred to selection media MS3C5RCK (10 explants per plate) and sealed before returning to the control temperature room for 2 weeks.

10

## Subculture of explants

Following selection explants were subcultured every 2 weeks on MS3C5ZRCK medium. When shoots developed they were carefully excised and transferred to Phytatrays (Sigma) containing MS3C5CK. DNA samples for PCR analysis were collected when shoots were sufficiently developed. Once the shoots rooted they were transferred to the glasshouse where initially they were placed in vermiculite with 1g/L Osmocote slow release fertiliser and then once roots were established they were transferred to soil.

15

20

## Constructs for transformation

25

pVB6\_35S-TSEC-LML and pVB6-TSEC-LML are shown in diagrammatic form in Figures 7 and 6 respectively.

## Analysis of transformants

30

1. All transformation were tested for the transgene, using PCR with *E.coli* Dxps-specific primers:

Forward: 5'-GCG CCG CTA TTT ACT CGA-3'

35

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Reverse: 5'TTT CTC TGG CGT GCC GCC-3'

2. Those that were PCR positive were tested by Southern blot analysis for the number of inserts, using the nptII probe.
3. Single insert transformation were then analysed for Dxps expression using RT-PCR, and the primers described in 1, above.
4. Expressing lines were tested for DXPS protein levels using Western blots with an antibody specific for the *E.coli* protein. A band ca. 69 kDa was found, showing both expression of transgene and cleavage of the transit peptide from the mature protein.
5. Seed was collected from all single insert lines for sowing.
6. T1 progeny were cultivated for pigment analysis and inheritance of phenotype.

Isoprenoids constitute a large group of compounds many of which are of high economic value. The condensation of (hydroxy)thiamin, derived from the decarboxylation of pyruvate, with glyceraldehyde-3-phosphate to yield 1-deoxy-D- xylulose-5-phosphate, is considered to be the first reaction in the mevalonate-independent pathway for IPP and ultimately isoprenoid biosynthesis. The data presented show that increasing the rate of DXP synthesis in *E. coli* results in increased isoprenoid biosynthesis. This finding can therefore be utilised to optimise the industrial production of isoprenoids from bacteria. The

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manipulation of enzyme activities important in the biosynthesis of specific isoprenoids in concert with DXPS may be employed to bioengineer the production of specific, high value isoprenoids in *E. coli* or another suitable cell or organism such as in plants where increased isoprenoid production could be used for improving crop flavour, fragrance and colour. Alternatively, crops could be engineered to produce increased concentrations of isoprenoids with pharmaceutical and/or nutritional properties.

**TABLE 1.** DXP synthase activity in *E. coli* homogenates

	<b>Specific activity</b> <b>nmol/min/mg protein</b>	<b>Fold increase</b> <b>in activity</b>
Control	5.8 $\pm$ 0.07	1.0
<i>B. subtilis</i>	11.5 $\pm$ 0.58	2.0
<i>Syn. sp.</i> 6803	10.4 $\pm$ 0.24	1.8

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**Claims**

1. A method of manipulating isoprenoid  
expression in a cell or organism having a mevalonate  
independent isopentyl diphosphate synthesising  
5 pathway, which method comprises altering the activity  
of the enzyme 1-deoxy-D-xylulose-5-phosphate synthase  
(DXPS), or a functional equivalent, derivative or  
bioprecursor thereof.

10

2. A method according to claim 1 wherein said  
isoprenoid production is increased by enhancing the  
activity or expression of said DXPS or lowered by  
inhibiting the activity or expression of said DXPS  
15 enzyme.

3. A method according to claim 2 wherein said  
enhanced DXPS activity occurs by transformation of  
said cell or organism with a vector comprising a  
20 nucleic acid molecule encoding said DXPS operably  
linked to an expression control sequence and  
optionally a reporter molecule

4. A method according to claim 3 wherein said  
25 DXPS encoded by said nucleic acid sequence is  
endogenous to said cell or organism.

5. A method according to claim 3 or 4 wherein  
said vector comprises one or more nucleic acid  
30 sequences encoding a polypeptide(s) capable of  
producing a desired isoprenoid in said cell or  
organism.

6. A method according to claim 3 or 4 wherein  
35 said cell or organism is transformed with a further

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vector comprising one or more nucleic acid sequences encoding a polypeptide(s) capable of producing a desired isoprenoid.

5           7. A method according to any preceding claim wherein said cell is any of a bacterial, yeast or algal cell.

8. A method according to claim 7 wherein said  
10 bacterial cell is *E. coli*.

9. A method according to any preceding claim wherein said organism is a plant.

15           10. A method according to any of claims 3 to 9 wherein said vector comprising said nucleic acid sequence(s) encoding said DXPS and/or said polypeptide(s) capable of producing said isoprenoid further comprises a nucleic acid sequence of either a  
20 tissue specific promoter and/or encoding a plastid transit peptide.

11. A method according to any of claims 5 to 10 wherein said desired isoprenoid is one conferring a  
25 nutritional benefit or an aesthetic phenotype.

12. A method according to claim 11 wherein said isoprenoid comprises any of the carotenoids, vitamins E, B1 or B6, chlorophylls, phenylquinones or  
30 diterpenes.

13. A cell or organism which has a mevalonate independent IPP biosynthetic pathway and which is transformed or transfected with a vector comprising a  
35 nucleic acid sequence encoding DXPS or a functional

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equivalent, derivative or bioprecursor thereof operably linked to an expression control sequence.

14. A cell or organism according to claim 13 wherein said vector further comprises a nucleic acid molecule encoding a reporter molecule.

15. A cell or organism according to claim 13 or 14 which further comprises a vector comprising one or more nucleic acid sequences encoding one or more polypeptides capable of producing a desired isoprenoid.

16. A cell or organism according to claim 15 wherein said desired isoprenoid comprises any of the carotenoids, vitamin E, B1 or B6, chlorophylls, phenylquinones, or diterpenes.

17. A method of identifying a compound which modulates isoprenoid activity or expression said method comprising contacting said compound to be tested with a molecule which is a component of the mevalonate independent IPP biosynthetic pathway and which molecule undergoes a reaction catalysed by DXPS activity in the presence of an appropriate reactant, in the presence of DXPS or a functional equivalent thereof and monitoring the yield of a product of the reaction when compared to the same reaction performed in the absence of the compound to be tested.

18. A method according to claim 17 wherein said molecule comprises pyruvate and said appropriate reactant comprises glyceraldehyde-3-phosphate or vice versa.

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19. A compound identified as a modulator of isoprenoid activity or expression according to the method of claim 17 or 18.

5        20. A compound according to claim 19 which comprises an inhibitor of DXPS or a functional equivalent of DXPS.

10       21. A compound according to claim 20 for use as a medicament or as a herbicide.

15       22. Use of a compound according to claim 20 in the preparation of a medicament to treat bacterial associated disease.

20       23. A pharmaceutical composition comprising a compound according to claim 20 together with a pharmaceutically acceptable carrier, diluent or excipient therefor.

24. A herbicidal composition comprising a compound according to claim 20.

25       25. A transgenic cell, tissue or organism having a mevalonate independent IPP biosynthetic pathway which cell, tissue or organism comprises at least one transgene capable of expressing DXPS or a functional equivalent, derivative or bioprecursor thereof.

30       26. A transgenic cell, tissue or organism according to claim 25, which comprises at least one additional copy of any of the nucleic acid sequences identified in Figure 3, or the complement thereof.

35       27. A transgenic cell, tissue or organism

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according to claim 25 or 26, further comprising a transgene capable of expressing one or more polypeptides capable of producing a desired isoprenoid, or a functional equivalent, derivative or bioprecursor thereof.

28. A transgenic cell, tissue or organism according to any of claims 25 to 27, wherein said organism is a plant.

29. A transgenic cell tissue or organism according to claim 28, wherein said plant is of the *Lycopersicon* spp.

30. Progeny of the organism according to any of claims 25 to 29.

31. A transformed plant comprising a transgene capable of expressing DXPS from *E.coli* having the sequence according to Figure 8 and which plant comprises a higher level of isoprenoid than an untransformed plant.

32. A transformed plant according to claim 31 comprising any of constructs pVB6\_TSEC\_LML or pVB6\_35S\_TSEC-LML illustrated in Figures 6 and 7.

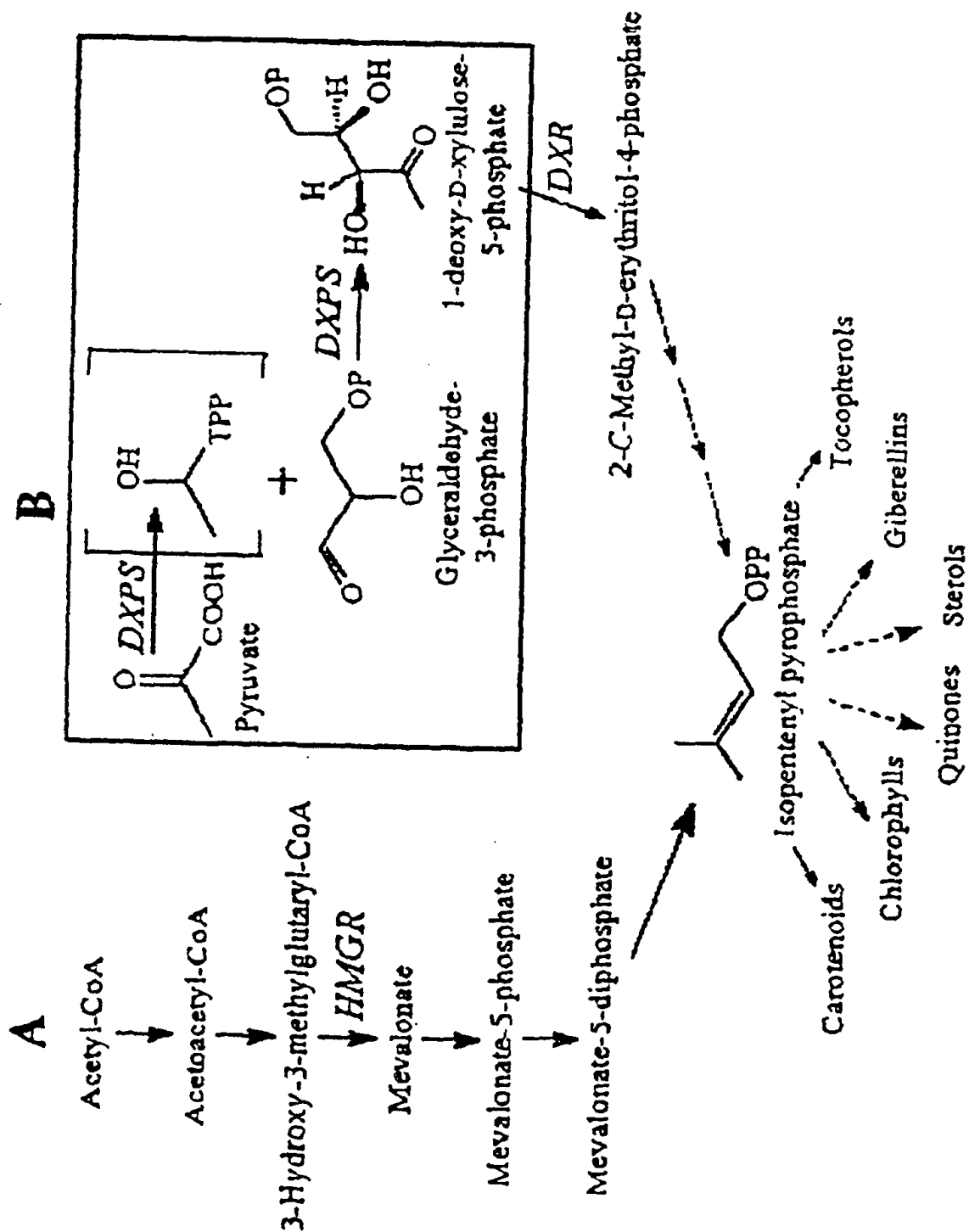
33. A transformed plant according to claim 31 or 32 wherein said plant is a tomato plant.

34. A tomato fruit produced by a plant according to claim 33.

35. A seed produced by a plant according to claim 33.

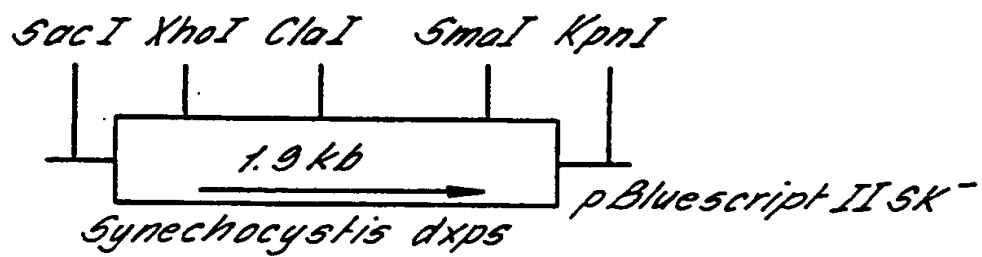
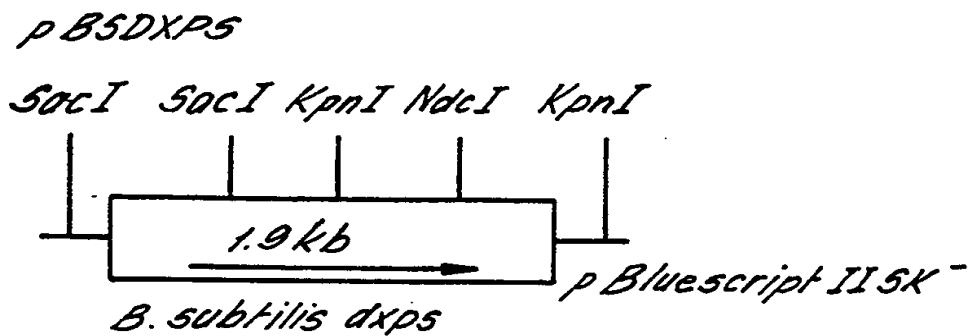
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FIG. 1



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FIG. 2.



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```

synxxx1 -----MDLLSIQDPSEFLKNMSIDELEKLSDEIRQFLITSLSASGGHIGPNLGVVELT
bacillus -----MDLLSIQDPSEFLKNMSIDELEKLSDEIRQFLITSLSASGGHIGPNLGVVELT
Ecolix0  MSFDIAKYPTLALVDSTQELRLLPKESLPKLCDELRRYLDSVSRSSGHFASGLGTVELT

synxxx1 VALHKEFNSEPKDFLWDVGHQSYVHKLITGRGKEFATLROYKGLCGFPKRSESEHDVWET
bacillus VALHKEFNSEPKDFLWDVGHQSYVHKLITGRGKEFATLROYKGLCGFPKRSESEHDVWET
Ecolix0  VALHYVYNTPEFDQLIWDVGHQAYEHRKLTGRDRDKIGTIRQKGLHFPFWRGSEYDVLSV

synxxx1 GHSSTSLSGAMGMAAARDIKGTDEYIIPITIGDGALTGGMALEALNHIGDEKKDMIVILND
bacillus GHSSTSLSGAMGMAAARDIKGTDEYIIPITIGDGALTGGMALEALNHIGDEKKDMIVILND
Ecolix0  GHSSTSISAGIGTAVAAEKEGKNRRTVCVIGDGAITAGMAFEAMNHAGDIRPDMIVILND

synxxx1 NEMSIAPNVGAIHSMGLRLRTAGKYQWVKDELEYLEKKIPAVGGKLAATAERVKDSLKYM
bacillus NEMSIAPNVGAIHSMGLRLRTAGKYQWVKDELEYLEKKIPAVGGKLAATAERVKDSLKYM
Ecolix0  NEMSISENVGALNNHLAQLLSGKLYSSLREGGKKVFSGVPPIKEILLKRTTEEHKIG----M

synxxx1 LVSGMFFFEELGETYLGPVDGHSYHELLENLOYAKKTRGPVLLHVITKKGKGYKPAETDTI
bacillus LVSGMFFFEELGETYLGPVDGHSYHELLENLOYAKKTRGPVLLHVITKKGKGYKPAETDTI
Ecolix0  VVPGTLEELGENTYIGPVDGHDVLGLITTLKNMRDLKGPQFLHIMTKKGRGYEPABKDFI

synxxx1 GTWHGTGPYKINTGDFVKPAAABSWSGLVSGTVORMAREDGRIVAITPAMPVGSKLEGF
bacillus GTWHGTGPYKINTGDFVKPAAABSWSGLVSGTVORMAREDGRIVAITPAMPVGSKLEGF
Ecolix0  -TFHAVPKFDPSSGCLPKSSGGIPSYSKIFGDWLCETAAKDNKLMAITPAMREGSGMVEF

synxxx1 AKEFPDRMFEDVGIAEQHAATMAAAMAMOGMKPFLAIYSTFLQRAYDQVVHDICRONANVF
bacillus AKEFPDRMFEDVGIAEQHAATMAAAMAMOGMKPFLAIYSTFLQRAYDQVVHDICRONANVF
Ecolix0  SRKFPDRYFDVAIAEQHAVTFAAGTAIGSYKPIVAIYSTFLQRAYDQVVLHDVATQKLPVVL

synxxx1 IGIDRAGLVGADGETHQGVFDIAFMRHIPNMVLMMPKDENEGQHMVHTALS YDEGPIAMR
bacillus IGIDRAGLVGADGETHQGVFDIAFMRHIPNMVLMMPKDENEGQHMVHTALS YDEGPIAMR
Ecolix0  FAIDRAGIVGADGQTHQGAFDLSYLRGCEPMVIMTPSDENECROMLYTGYHYNDGFSAVR

synxxx1 FPRGNGLGVKMDQOLKTIPIGTWEVLRPGNDVILTFGTTIEMAIEAAEELQKEGLSVRV
bacillus FPRGNGLGVKMDQOLKTIPIGTWEVLRPGNDVILTFGTTIEMAIEAAEELQKEGLSVRV
Ecolix0  YPRGNAGVVEITP-LEKLPIGKGIKRRRGEKLAILNFGTLMPEAAKVAESLN-----ATL

synxxx1 VNARFIKPIDEKMMKSILKEGLPILTTEEAVLEGCGSSILEFAHDQGEYHTPIDRMGIP
bacillus VNARFIKPIDEKMMKSILKEGLPILTTEEAVLEGCGSSILEFAHDQGEYHTPIDRMGIP
Ecolix0  VDMRFVKPIDEALILEMAASHEALVTVEENAIMGGAGSGVNEVLMMAHR-KPVVPLNIGLP

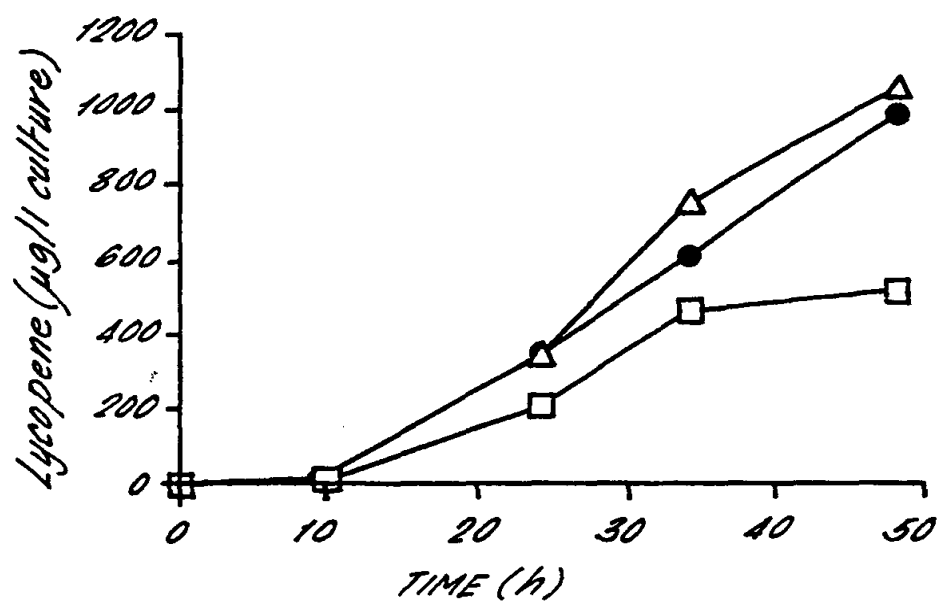
synxxx1 DREIEHGSVTALLEEIGLTKQOVANRIRLLMPPKTHKGIGS
bacillus DREIEHGSVTALLEEIGLTKQOVANRIRLLMPPKTHKGIGS
Ecolix0  DFFIIPQGTQEEMRAELGLDAAGMEAKIKAWLA-----

```

FIG. 3.

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FIG. 4.



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FIG. 5.

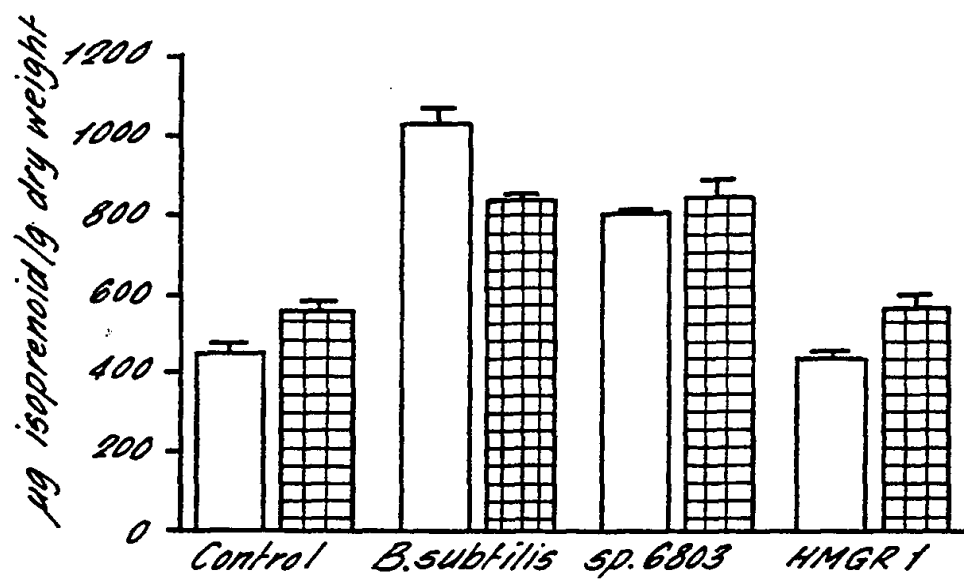
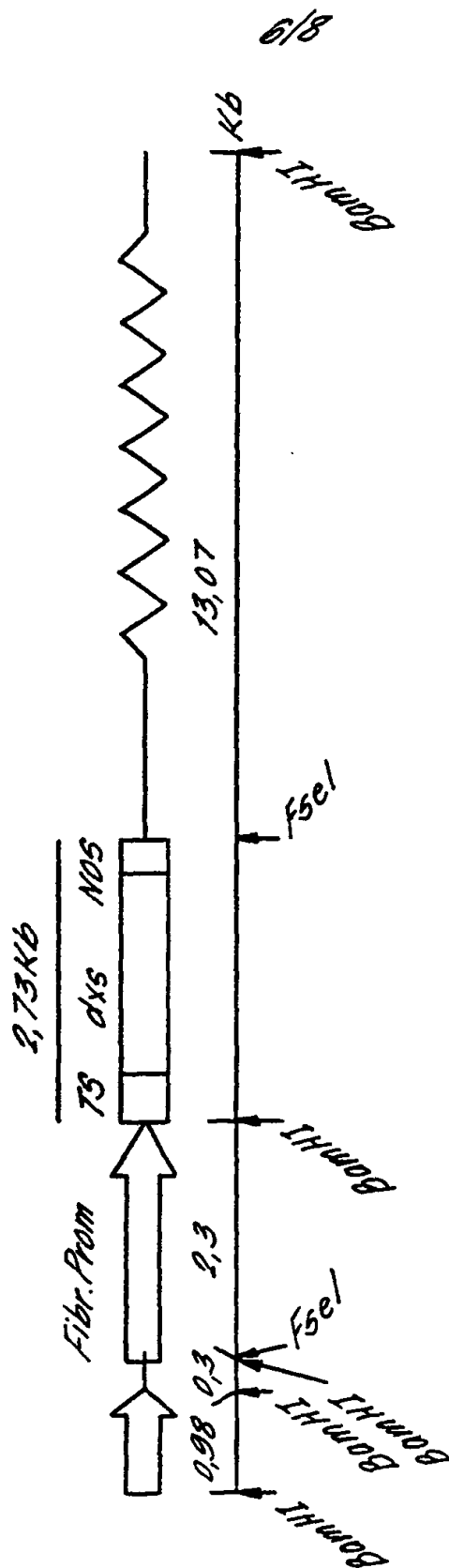
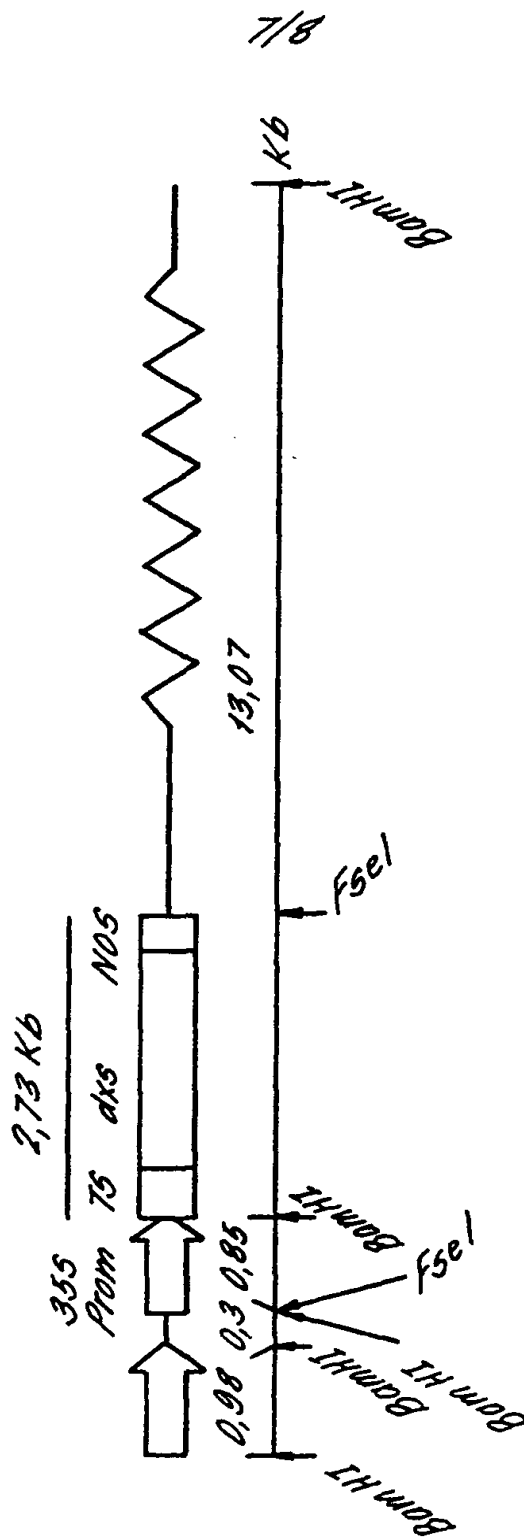


FIG. 6.



Construction name: pVB6\_TSEC\_LML  
 Fibr. Prom: Fibrillin promoter  
 T5: transit sequence  
 dxs: deoxyxylulose synthase gene from *E. coli*  
 Date: October 1998

FIG. 7.



Construction name: pVB6\_35S\_TSFC\_LML  
 35S Prom: 35S promoter  
 TS: transit sequence  
 dxs: deoxyxylulose synthase gene from *E. coli*  
 Date: October 1998

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FIG. 8.

msfdiakypptlalvdstqelrlpkesspkcldelrrylldsvsrssghfasglgtveltvalhyvyntpfdqliwdvghqa  
 yphkiltgrrdkigtirqkgglhpfwrgeseydvlsvglhsstsisagigiavaaekgknrtvcvigdgaitagmfe  
 amnhagdirpdmilvilndnemsisenvgalnnhlaqlsgklysslreggkkvfsgvppikellkrteehikgmvv  
 gtlfeelgfnyigpvdghdvlglittlknmrdlkgpqflhimtkkgrgyepaekdpitfhavpkfdpssgclpkssgglp  
 syskifgdwlcetaakdnklmaitpamregsgmvfssrkfpdryfdvaiaeqhavlfaaglaiggykpivaiystflqr  
 aydqvldvaiqlpvlfaidragivgadgqthqgafdisylrcipemvimtpsdeneqrmlytgyhyndgpsavr  
 yprgnavgveltpleklpigkgivkrrgeklailnfgtlmpeaakvaesnatlvdmrfvkldealilemaashealvt  
 veenaimggagsgvnevlmahrkpvplniglpdffipqgtqeemraelgladaagmeakikawla

FIG. 9.

malcayafpgilnrtgvvsdsskatplfsgwihgtdlqflfqhklthevkkrsrvvqaslsesgeyytqrptpildtvny  
 pihmknslskelqkladelrsdtifnvsktgghlgsslgvvveltvalhyvfnapqdriildvghqsyphkiltgrrdkms  
 tlrqtdglagftrseseydcfg

# PCT

## REQUEST

The undersigned requests that the present international application be processed according to the Patent Cooperation Treaty.

For receiving Office use only

International Application No.

International Filing Date

Name of receiving Office and "PCT International Application"

Applicant's or agent's file reference  
(if desired) (12 characters maximum) SCB/52068001

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<b>Box No. II APPLICANT</b>	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.) ROYAL HOLLOWAY AND BEDFORD NEW COLLEGE UNIVERSITY OF LONDON EGHAM SURREY TW20 0EX UNITED KINGDOM	
<input type="checkbox"/> This person is also inventor.	
Telephone No. 01784 443559	
Facsimile No. 01784 434326	
Teleprinter No.	
State (that is, country) of nationality: UK	State (that is, country) of residence: UK
This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input checked="" type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box	
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This person is: <input type="checkbox"/> applicant only <input checked="" type="checkbox"/> applicant and inventor <input type="checkbox"/> inventor only (If this check-box is marked, do not fill in below.)	
State (that is, country) of nationality: UK	State (that is, country) of residence: UK
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<input checked="" type="checkbox"/> Further applicants and/or (further) inventors are indicated on a continuation sheet.	
<b>Box No. IV AGENT OR COMMON REPRESENTATIVE; OR ADDRESS FOR CORRESPONDENCE</b>	
The person identified below is hereby/has been appointed to act on behalf of the applicant(s) before the competent International Authorities as: <input checked="" type="checkbox"/> agent <input type="checkbox"/> common representative	
Name and address: (Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country.) BOULT WADE TENNANT 27 FURNIVAL STREET LONDON EC4A 1PQ UNITED KINGDOM	
Telephone No. +44 (0)20 7430 7500	
Facsimile No. +44 (0)20 7831 1768	
Teleprinter No.	
<input type="checkbox"/> Address for correspondence: Mark this check-box where no agent or common representative is/has been appointed and the space above is used instead to indicate a special address to which correspondence should be sent.	

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State <i>(that is, country)</i> of nationality: UK	State <i>(that is, country)</i> of residence: UK
<p>This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input checked="" type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box</p>	
<p>Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i></p>	<p>This person is:</p> <p><input type="checkbox"/> applicant only</p> <p><input type="checkbox"/> applicant and inventor</p> <p><input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i></p>
State <i>(that is, country)</i> of nationality:	State <i>(that is, country)</i> of residence:
<p>This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box</p>	
<p>Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i></p>	<p>This person is:</p> <p><input type="checkbox"/> applicant only</p> <p><input type="checkbox"/> applicant and inventor</p> <p><input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i></p>
State <i>(that is, country)</i> of nationality:	State <i>(that is, country)</i> of residence:
<p>This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box</p>	
<p>Name and address: <i>(Family name followed by given name; for a legal entity, full official designation. The address must include postal code and name of country. The country of the address indicated in this Box is the applicant's State (that is, country) of residence if no State of residence is indicated below.)</i></p>	<p>This person is:</p> <p><input type="checkbox"/> applicant only</p> <p><input type="checkbox"/> applicant and inventor</p> <p><input type="checkbox"/> inventor only <i>(If this check-box is marked, do not fill in below.)</i></p>
State <i>(that is, country)</i> of nationality:	State <i>(that is, country)</i> of residence:
<p>This person is applicant for the purposes of: <input type="checkbox"/> all designated States <input type="checkbox"/> all designated States except the United States of America <input type="checkbox"/> the United States of America only <input type="checkbox"/> the States indicated in the Supplemental Box</p>	
<p><input type="checkbox"/> Further applicants and/or (further) inventors are indicated on another continuation sheet.</p>	

## Box No. V DESIGNATION OF STATES

The following designations are hereby made under Rule 4.9(a) (mark the applicable check-boxes; at least one must be marked):

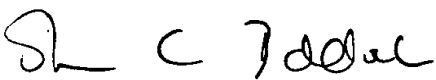
## Regional Patent

- ☒ AP ARIPO Patent: GH Ghana, GM Gambia, KE Kenya, LS Lesotho, MW Malawi, SD Sudan, SL Sierra Leone, SZ Swaziland, UG Uganda, ZW Zimbabwe, and any other State which is a Contracting State of the Harare Protocol and of the PCT
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National Patent (if other kind of protection or treatment desired, specify on dotted line):

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| <input checked="" type="checkbox"/> AM Armenia                              | <input checked="" type="checkbox"/> LT Lithuania   |
| <input checked="" type="checkbox"/> AT Austria                              | <input checked="" type="checkbox"/> LU Luxembourg  |
| <input checked="" type="checkbox"/> AU Australia                            | <input checked="" type="checkbox"/> LV Latvia  |
| <input checked="" type="checkbox"/> AZ Azerbaijan                           | <input checked="" type="checkbox"/> MD Republic of Moldova   |
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| <input checked="" type="checkbox"/> BB Barbados                             | <input checked="" type="checkbox"/> MK The former Yugoslav Republic of Macedonia                             |
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| <input type="checkbox"/> HR Croatia   | <input type="checkbox"/> TR Turkey   |
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| <input type="checkbox"/> KG Kyrgyzstan                                      | <input type="checkbox"/> YU Yugoslavia   |
| <input type="checkbox"/> KP Democratic People's Republic of Korea           | <input checked="" type="checkbox"/> ZA South Africa  |
|   | <input type="checkbox"/> ZW Zimbabwe   |
| <input type="checkbox"/> KR Republic of Korea                               | Check-boxes reserved for designating States which have become party to the PCT after issuance of this sheet: |
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| <input type="checkbox"/> LK Sri Lanka                                       | <input type="checkbox"/> DM Dominica   |
|   | <input type="checkbox"/> MA Morocco  |

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<b>Box No. VI PRIORITY CLAIM</b>		<input type="checkbox"/> Further priority claims are indicated in the Supplemental Box.		
Filing date of earlier application (day/month/year)	Number of earlier application	Where earlier application is:		
		national application: country	regional application:* regional Office	international application: receiving Office
item (1) 28 JANUARY 1999	9901902.8	UNITED KINGDOM		
item (2)				
item (3)				
<input type="checkbox"/> The receiving Office is requested to prepare and transmit to the International Bureau a certified copy of the earlier application(s) (only if the earlier application was filed with the Office which for the purposes of the present international application is the receiving Office) identified above as item(s): _____				
<small>* Where the earlier application is an ARIPO application, it is mandatory to indicate in the Supplemental Box at least one country party to the Paris Convention for the Protection of Industrial Property for which that earlier application was filed (Rule 4.10(b)(ii)). See Supplemental Box.</small>				
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<b>Choice of International Searching Authority (ISA)</b> <small>(if two or more International Searching Authorities are competent to carry out the international search, indicate the Authority chosen; the two-letter code may be used):</small>		<b>Request to use results of earlier search; reference to that search</b> (if an earlier search has been carried out by or requested from the International Searching Authority):		
ISA / EP		Date (day/month/year)      Number      Country (or regional Office)		
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This international application contains the following number of sheets: request : 4 description (excluding sequence listing part) : 33 claims : 5 abstract : 1 drawings : 8 sequence listing part of description : 0 Total number of sheets : 51		This international application is accompanied by the item(s) marked below: 1. <input checked="" type="checkbox"/> fee calculation sheet 2. <input type="checkbox"/> separate signed power of attorney 3. <input type="checkbox"/> copy of general power of attorney; reference number, if any: 4. <input type="checkbox"/> statement explaining lack of signature 5. <input type="checkbox"/> priority document(s) identified in Box No. VI as item(s): 6. <input type="checkbox"/> translation of international application into (language): 7. <input type="checkbox"/> separate indications concerning deposited microorganism or other biological material 8. <input type="checkbox"/> nucleotide and/or amino acid sequence listing in computer readable form 9. <input type="checkbox"/> other (specify):		
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<b>Box No. IX SIGNATURE OF APPLICANT OR AGENT</b>				
<small>Next to each signature, indicate the name of the person signing and the capacity in which the person signs (if such capacity is not obvious from reading the request).</small>				
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28 January 2000				

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International application No.

Applicant's or agent's  
file reference SCB/52068/001

Date stamp of the receiving Office

Applicant

ROYAL HOLLOWAY AND BEDFORD NEW COLLEGE UNIVERSITY OF LONDON

### CALCULATION OF PRESCRIBED FEES

1. TRANSMITTAL FEE . . . . . £55 T

2. SEARCH FEE . . . . . £638 S

International search to be carried out by EP  
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### 3. INTERNATIONAL FEE

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The international application contains 51 sheets.

first 30 sheets . . . . . £264 b1

21 x £6 = £126 b2

remaining sheets additional amount

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SCB/52068001

## IMPORTANT NOTIFICATION

International application No.  
PCT/GB00/00263International filing date (day/month/year)  
28/01/2000Priority date (day/month/year)  
28/01/1999 -Applicant  
ROYAL HOLLOWAY AND BEDFORD NEW COLLEGE et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.
2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.
3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

## 4. REMINDER

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the International Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/



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PCT/GB00/00263

# PCT

## INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference SCB/52068001		<b>FOR FURTHER ACTION</b> See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
International application No. PCT/GB00/00263	International filing date (day/month/year) 28/01/2000	Priority date (day/month/year) 28/01/1999
International Patent Classification (IPC) or national classification and IPC C12N15/54		
Applicant ROYAL HOLLOWAY AND BEDFORD NEW COLLEGE et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.


2. This REPORT consists of a total of 12 sheets, including this cover sheet.

☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of 5 sheets.

3. This report contains indications relating to the following items:

- I ☒ Basis of the report
- II ☒ Priority
- III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV ☒ Lack of unity of invention
- V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI ☒ Certain documents cited
- VII ☒ Certain defects in the international application
- VIII ☒ Certain observations on the international application

Date of submission of the demand  02/06/2000	Date of completion of this report  02.05.2001
Name and mailing address of the international preliminary examining authority:   European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer  Rojo Romeo, E  Telephone No. +49 89 2399 7321



**INTERNATIONAL PRELIMINARY  
EXAMINATION REPORT**

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**I. Basis of the report**

1. With regard to the **elements** of the international application (*Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17)*):

**Description, pages:**

1-33 as originally filed

**Claims, No.:**

1-34 as received on 29/03/2001 with letter of 28/03/2001

**Drawings, sheets:**

1/8-8/8 as originally filed

**Sequence listing part of the description, pages:**

1-16, filed with the letter of 08.05.00

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

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- ☐ the description,                      pages:
- ☐ the claims,                              Nos.:
- ☐ the drawings,                          sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

*(Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)*

6. Additional observations, if necessary:

## II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

- ☐ copy of the earlier application whose priority has been claimed.
- ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:  
**see separate sheet**

### III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

- ☐ the entire international application.
- ☒ claims Nos. 22, 23.

**because:**

- ☐ the said international application, or the said claims Nos. relate to the following subject matter which does not require an international preliminary examination (*specify*):
- ☒ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. 22, 23 are so unclear that no meaningful opinion could be formed (*specify*):  
**see separate sheet**

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- ☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.
- ☐ no international search report has been established for the said claims Nos. .
2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:
- ☐ the written form has not been furnished or does not comply with the standard.
- ☐ the computer readable form has not been furnished or does not comply with the standard.

**IV. Lack of unity of invention**

1. In response to the invitation to restrict or pay additional fees the applicant has:
- ☐ restricted the claims.
- ☐ paid additional fees.
- ☐ paid additional fees under protest.
- ☐ neither restricted nor paid additional fees.
2. ☒ This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is
- ☐ complied with.
- ☒ not complied with for the following reasons:  
**see separate sheet**
4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:
- ☒ all parts.
- ☐ the parts relating to claims Nos. .

**V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Statement

Novelty (N)	Yes: Claims 1-21, 26, 30-32
	No: Claims 24, 25, 27-29, 33, 34
Inventive step (IS)	Yes: Claims 1-4, 8, 9, 18, 21, 30, 31, 32

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	No:	Claims	5-7, 10-17, 19, 20, 24-29, 33, 34
Industrial applicability (IA)	Yes:	Claims	1-34
	No:	Claims	

2. Citations and explanations  
**see separate sheet**

**VI. Certain documents cited**

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

**see separate sheet**

**VII. Certain defects in the international application**

The following defects in the form or contents of the international application have been noted:  
**see separate sheet**

**VIII. Certain observations on the international application**

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:  
**see separate sheet**

**R Item I**

**Basis of this report**

Concerning the following comments, the Applicant's arguments filed with his letter of 28.03.01 were carefully considered.

The amended set of claims is acceptable under Art. 19(2) PCT.

**Re Item II**

**Priority**

No right of priority can be acknowledged for claims addressed to vectors pVB6\_TSCE\_LML (Fig. 6), pVB6\_35S\_TSEC-LML (Fig. 7) or the transit peptide of Fig. 9, since none of these are disclosed in the priority document.

**R Item III**

**Non-establishment of opinion with regard to novelty, inventive step and industrial applicability**

1. Contrary to Applicant's assumptions that the method claim 22 (old claim 17) and dependent claim 23 (old claim 18) are clearly defined, the IPEA considers that these claims do not meet the requirements of Art. 6 PCT in that the matter for which protection is sought is not clearly defined. The claims attempt to define the subject-matter in terms of the result to be achieved which merely amounts to a statement of the underlying problem instead of defining the subject-matter in terms of technical features.
2. Consequently, claims 22, 23 are not examined.

**R Item IV**

**Lack of unity of invention**

Reference is made to the following documents cited in the International Search Report:  
D1: LOIS LUISA MARIA ET AL: 'Cloning and characterization of a gene from Escherichia coli encoding a transketolase-like enzyme that catalyzes the synthesis of D-1-deoxy-xylulose 5-phosphate, a common precursor for isoprenoid, thiamin, and pyridoxol biosynthesis.' PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES OF AMERICA MARCH 3, 1998, vol. 95, no. 5, 3 March 1998 (1998-03-03), pages 2105-2110, XP002116673 ISBN: 0027-8424 cited in the

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- D2: LANGE ET AL: 'A family of transketolases that directs isoprenoid biosynthesis via a mevalonate-independent pathway' FASEB JOURNAL,US,FED. OF AMERICAN SOC. FOR EXPERIMENTAL BIOLOGY, BETHESDA, MD, vol. 95, March 1998 (1998-03), pages 2100-2104, XP002116672 ISSN: 0892-6638 cited in the application
- D3: KANEKO, T., ET AL. : 'sequence analysis of the genome of the unicellular cyanobacterium Synechocystis sp. strain PCC6803. II. sequence determination of the entire genome and assignment of potential protein-coding regions' EMBL SEQUENCE DATA LIBRARY, 15 July 1998 (1998-07-15), XP002139910 heidelberg, Germany
- D4: KOBAYASHI, Y., ET AL. : 'untitled' EMBL SEQUENCE DATA LIBRARY, 1 October 1996 (1996-10-01), XP002139911 heidelberg, Germany

According to Rule 13 PCT an application shall relate to one invention only or to a group of inventions so linked as to form a single general inventive concept, i.e. having at least one common technical feature defining a contribution over the known prior art.

The common inventive concept linking the present claims is the provision and use of a nucleic acid molecule encoding a 1-deoxy-D-xylulose-5-phosphate synthase to enhance the activity of this enzyme in cells which have a mevalonate independent IPP biosynthetic pathway. In the prior art, several 1-deoxy-D-xylulose-5-phosphate synthases have been described (see D1-D4) and the corresponding nucleic acids have been cloned. Vectors containing, e.g. the gene of the E.coli, were used for bacterial transformation and manipulating the expression of 1-deoxy-D-xylulose-5-phosphate synthase, thus enhancing the activity of this enzyme 150 times (see D1, page 2106, right column; page 2109, right column). Thus, this common inventive concept no longer exists and each and every 1-deoxy-D-xylulose-5-phosphate synthase defines an independent invention (1-deoxy-D-xylulose-5-phosphate synthase from E.coli, B. subtilis, Synechocystis sp.; see claim 26).

Consequently, in the regional phase, an objection for lack of unity may arise.

**Re Item V**

**Reasoned statement under Rule 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement**

1. Novelty (Art. 33(2) PCT)

- 1.1 D1 discloses the protein sequence of the 1-deoxy-D-xylulose-5-phosphate synthase of *E. coli* and the alignment of this sequence with the amino acid sequences from *H. influenza*, *R. capsulatus*, *Synechocystis p.*, *B. subtilis*, *H. pylori*, *M. tuberculosis*, *A. thaliana*, *Mentha x piperita* (see Fig. 3). The corresponding gene was cloned and expressed in *E. coli*. Induced over-expression of the gene for 1-deoxy-D-xylulose-5-phosphate synthase led to a 250-fold enhanced activity of this enzyme.

Consequently, D1 anticipates the subject-matter of claims 24, 25, 27-29 (the cell could be a bacteria).

- 1.2 Concerning claims 29, 33 and 34, the progeny (fruit or seeds) of a plant does not necessarily have all genetic characteristics of the mother plant. Therefore, in the absence of a technical feature distinguishing the claimed fruit (or seeds) from known plant (in particular tomato) fruit and seeds, the subject-matter of claims 29, 33 and 34 cannot be acknowledged novelty. The Applicant's attention is drawn to the fact that the feature "having increased isoprenoid activity" is not sufficient to characterise the progeny of a transformed plant since it is not clear whether said progeny (whether fruit or seed or plant) carries the gene of a bacterial DXPS. Thus, any plant having naturally an increased isoprenoid level compared to any other plant would be covered by the scope of these claims.
- 1.3 In summary, claims 24, 25, 27-29 (partially), 33 and 34 are not novel, and thus, not inventive.

2. Inventive step (Art. 33(3) PCT)

- 2.1 Claims 14-17, 19, 20 are directed to a method in "a cell" which could be a bacteria. A bacteria (e.g. *E. coli*) transformed with a gene encoding a DXPS is not novel (see above). The addition of a transformation step with a vector without any technical characteristics which could bring an advantage over the method disclosed in D1 does not establish inventive activity. Thus, claims 14-17, 19, 20 lack inventive activity.
- 2.2 The subject-matter of claim 26 is considered to be an obvious embodiment of claim

24 which is not novel. Thus, this claim lacks inventive activity.

- 2.3 Concerning claims 5, 6, 7, 12, 13, 14 (and dependent claims) and 27 (partially), there is no hint in the prior art to transform cells with (at least) both the DXPS and another gene encoding one (or more) polypeptides capable of producing a desired isoprenoid. However, the Applicant's attention is drawn to the fact, that, in the absence of proper support by the specification (see VIII, 2.1), no inventive activity can be acknowledged for the subject-matter of these claims.
- 2.4 As far as claim 10 and dependent claims 11-13 are concerned, inventive activity could only be acknowledged once the objections raised under VIII, 1.1 and 1.2 are relieved.
- 2.5 Concerning claims 1-4, 8, 9, 30, 31, 32, the Applicant's attention is drawn to the fact, that in document D2, it is suggested to use "the mevalonate-independent pathway (...) as a novel approach to transgenic manipulation of plant isoprenoid biosynthesis, and, because this new pathway is present in bacteria and plants but not in animals, it provides a unique target for the design of highly specific antibiotics and herbicides". However, no specific indication is found for the transgenic expression of DXPS (in particular from bacteria) into plants. Consequently, inventive activity could be acknowledged for the subject-matter of these claims.
- 2.6 Similarly, claims 18 and 21 could be acknowledged inventive activity, once the objection for lack of clarity are alleviated (see VIII, 1.3).

Consequently, 5, 6, 7, 10-17, 19, 20, 26 claims lack inventive step.

#### **R Item VI**

##### **Certain documents cited**

D5: WO 99 58649 A (UNIV MARYLAND ;DELLAPENNA DEAN (US); MOEHS CHARLES P (US); CUNNING) 18 November 1999 (1999-11-18)

international publication date: 18.11.99

international filing date: 13.05.99

priority data: 13.05.98

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D6: WO 99 11757 A (MCCASKILL DAVID G ;LANGE BERND M (US); UNIV WASHINGTON (US); WILDU) 11 March 1999 (1999-03-11)

international publication date: 11.03.99

international filing date: 01.09.98

priority data: 02.09.97

D7: WO 99 52938 A (HASSAN JOMAA) 21 October 1999 (1999-10-21)

international publication date: 21.10.99

international filing date: 13.04.99

priority data: 14.04.98, 09.06.98, 24.06.98, 15.07.98, 22.09.98

D8: WO 99 53071 A (KUZUYAMA TOMOHISA ;MIYAKE KOICHIRO (JP); OZAKI AKIO (JP); SETO HAR) 21 October 1999 (1999-10-21)

international publication date: 21.10.99

international filing date: 14.04.99

priority data: 14.04.98, 05.08.98, 15.02.99

D9: WO 00 08169 A (EBNETH MARCUS ;HERBERS KARIN (DE); REINDL ANDREAS (DE); SUNGENE GM) 17 February 2000 (2000-02-17)

international publication date: 17.02.00

international filing date: 30.07.99

priority data: 05.08.98, 01.10.98

**R Item VII**

**C rtain defects in the international application**

1. The first two sequences of the alignment presented in Fig. 3 are identical and correspond to the sequence of DXPS from *B. subtilis* (see D1, Fig. 3). It is thus questionable whether the sequence from *Synechocystis* sp. is disclosed by the present application.
2. Some figures of the present application use the nomenclature DXPS, others DXS. This brings a certain inconsistency.
3. The Applicant's attention is drawn to the fact that he has to deposit the biological material corresponding to the vectors claimed pursuant to Rule 13bis.6. Conversely

to the Applicant's assumptions, the IPEA considers that the present specification does not provide enough information to lead to the exact same (i.e. identical nucleotide sequence) claimed vectors.

4. Claims 20 and 21 are redundant with claims 17 and 18, respectively.

**Re Item VIII**

**Certain observations on the international application**

1. Clarity (Art. 6 PCT)

- 1.1 The Applicant's attention is drawn to the fact that the current claims 10-16, 22-34 do not refer to a particular sequence to define the term DXPS. In the absence of a reference to a concrete sequence characterizing the DXPS protein or polynucleotide, respectively, said claims lack technical features necessary to clearly define the claimed-subject-matter since the term DXPS has no technical meaning for the person skilled in the art. Consequently, the said claims may be interpreted as being directed to any protein or polynucleotide.

Relating to this, the Applicant's attention is drawn to the fact that the claims must be clear without the context of the application.

In the interest of the Applicant, this report was drawn as if reference to the claimed nucleotide or amino acid sequence of DXPS had been made. Concerning this point, the Applicant is reminded that he has to take particular care and fulfill the requirements of Rule 70.2(c) PCT.

- 1.2 Concerning claim 10 and dependent claims thereof, the Applicant's attention is drawn to the fact that every protein can be considered to be a "derivative" of another protein by amino acid substitution, mutation... Therefore, these claims may be interpreted as being directed to any known protein and an objection for lack of novelty may arise. Moreover, the term "bioprecursor" has no technical meaning for the person skilled in the art, and thus, brings ambiguity to the subject-matter of these claims.
- 1.3 Claim 14 is unclear since the first vector transformed into the cell or organism is not defined and could contain any nucleic acid ("comprising a nucleic acid").

Moreover, the optional features seemed to be incomplete ("operably ... a reporter

molecule").

2. Support by specification (Art. 6 PCT), in combination with Art. 5 PCT (complete and enabling disclosure)
- 2.1 Concerning claims 5, 6, 7, 12, 14, 26, and dependent claims thereof, no indication is given in the specification as far as which genes are to be used (in addition to that encoding the already known DXPS enzyme(s)). Thus, the person skilled in the art would be left guessing and could not find out which genes of this new pathway are to be used without undue burden. Thus, the subject-matter of said claims is not supported by the specification and an objection for incomplete disclosure of the invention also arises.